



UNIVERSITY  
OF TASMANIA

# **Regulation of xylem ion loading and ionic relations in barley and wheat leaves in the context of salinity stress tolerance**

by

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Submitted in fulfilment of the requirements for the Doctor of Philosophy

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## ***Declaration of Originality***

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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December 2015

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Published in *Journal of Agronomy and Crop Science* doi: 10.1111/jac.12122;  
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**Paper 3: Physiological and molecular mechanisms mediating xylem Na<sup>+</sup> loading in barley in the context of salinity stress tolerance**

Submitted to *Plant Cell and Environment*; *Located in Chapter 4*

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**Paper 4: Nax loci affect SOS1-like Na<sup>+</sup>/H<sup>+</sup> exchanger expression and activity in wheat**

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## *Preliminaries*

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### **Abstract**

Soil salinization is the accumulation of water-soluble salts in the soil to a level that impacts on agricultural production. Approximately 20% of the world's cultivated land, or 6% of the world total area, is threatened by salinity. Hence, soil salinization is becoming the main challenge of the modern agriculture. Of all the cereals, wheat (*Triticum aestivum*) is a moderately salt-tolerant crop while barley (*Hordeum vulgare*) is classified as relatively salt tolerant. Within *Triticum* genera, durum wheat (*Triticum turgidum ssp. durum*) is less salt-tolerant than bread wheat (*Triticum aestivum*). Salinity tolerance is a complex trait, both physiologically and genetically. Both rapid and efficient osmotic adjustment and effective control of  $\text{Na}^+$  delivery to the shoot are absolutely critical for salinity stress tolerance. Reducing  $\text{Na}^+$  delivery from root to shoot can be achieved by either minimizing entry of  $\text{Na}^+$  to the xylem from the root symplast or maximizing  $\text{Na}^+$  retrieval from the xylem (e.g. by active means) or by reduced rate of transpiration resulted from stomata closure (e.g. by passive means). Specific details of their coordination and the relative contribution of these components towards salinity stress tolerance in wheat and barley have been not fully revealed until now.

Hence, the major aim of this PhD project was to investigate the physiological and molecular mechanisms of regulating  $\text{Na}^+$  transport from root to shoot and its link with plant osmotic adjustment and the overall plant performance under saline conditions. The following specific objectives were addressed:

- To quantify the relative contribution of organic and inorganic osmolytes towards osmotic adjustment in barley
- To link osmotic adjustment and stomatal characteristics with salinity stress tolerances in contrasting barley accessions
- To evaluate predictive values of various physiological indices for salinity stress tolerance in wheat and barley
- To investigate the physiological and molecular mechanisms mediating xylem  $\text{Na}^+$  loading in wheat and barley

## Abstract

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Working along these lines, a broad range of barley (*Hordeum vulgare* and *Hordeum spontaneum*) genotypes contrasting in salinity stress tolerance were used to investigate the causal link between plant stomatal characteristics, tissue ionic relations, and salinity tolerance. In total, 46 genotypes (including two wild barleys) were grown under glasshouse conditions and exposed to a moderate salinity stress (200 mM NaCl) for five weeks. The overall salinity tolerance correlated positively with stomata density, leaf  $K^+$  concentration and the relative contribution of inorganic ions towards osmotic adjustment in the shoot. At the same time, no correlation between salinity tolerance and stomatal conductance or leaf  $Na^+$  content in shoot was found. Taken together, these results indicate the importance of increasing stomata density as an adaptive tool to optimise efficiency of  $CO_2$  assimilation under moderate saline conditions, as well as benefits of the predominant use of inorganic osmolytes for osmotic adjustment, in barley. Another finding of note was that wild barleys showed rather different strategies dealing with salinity, as compared with cultivated varieties.

Then, a large number of wheat (*Triticum aestivum* and *Triticum turgidum*) cultivars were screened by using a broad range of physiological indices, to evaluate predictive values of various physiological indices for salinity stress tolerance in wheat cultivars. In general, most of the bread wheats showed better  $Na^+$  exclusion that was associated with higher relative yield. Leaf  $K^+/Na^+$  ratio and leaf and xylem  $K^+$  contents were the major factors determining salinity stress tolerance in wheat. Other important traits included high xylem  $K^+$  content, high stomatal conductance, and low osmolality. Bread wheat and durum wheat showed different tolerance mechanisms, with leaf  $K^+/Na^+$  content in durum wheat making no significant contributions to salt tolerance, while the important traits were leaf and xylem  $K^+$  contents. These results indicate that  $Na^+$  sequestration ability is much stronger in durum compared with bread wheat, most likely as a compensation for its lesser efficiency to exclude  $Na^+$  from transport to the shoot.

Based on the large screening of barley genotypes for their ability to exclude  $Na^+$  for its loading into the xylem and delivery to the shoot, four genotypes contrasting in salinity stress tolerance were selected for further studies of molecular and

## Abstract

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physiological mechanisms mediating xylem Na<sup>+</sup> and K<sup>+</sup> loading and linking it with overall plant performance and sequestration of Na<sup>+</sup> and K<sup>+</sup> in leaf tissues. We report that both leaf and xylem K<sup>+</sup>/Na<sup>+</sup> ratios correlated positively with overall plant salt tolerance after prolonged (3 weeks) exposures to salinity stress. Interestingly, it was Na<sup>+</sup> but not K<sup>+</sup> content that determined this correlation. At the same time, it was found that accumulation of Na<sup>+</sup> in xylem sap in salt-tolerant genotypes (TX9425 and CM72) reached a peak 5 days after salt application and then declined. In contrast, salt-sensitive genotypes were less efficient in controlling xylem Na<sup>+</sup> loading and showed a progressive increase in xylem Na<sup>+</sup> concentrations. We then used the MIFE (microelectrode ion flux measurement) technique to study some aspects of salt stress signalling and Na loading into the xylem. This was achieved by measuring net fluxes of Ca<sup>2+</sup>, K<sup>+</sup> and Na<sup>+</sup> from xylem parenchyma tissue of control- and salt-grown plants in response to a range of known second messengers such as H<sub>2</sub>O<sub>2</sub>, ABA, or cGMP. Our results indicate that NADPH oxidase-mediated apoplastic H<sub>2</sub>O<sub>2</sub> production acts upstream of xylem Na<sup>+</sup> loading and is causally related to ROS-inducible Ca<sup>2+</sup> uptake systems in the xylem parenchyma tissue. ABA was also able, directly or in-directly, to regulate the process of Na<sup>+</sup> retrieval from xylem. The above findings were further supported by molecular experiments revealing that salt-tolerant barley genotypes (CPI and CM72) upregulate transcript levels of *HvHKT1;5* and *HvSOS1* to take up Na<sup>+</sup> and transport them to shoot for osmotic adjustment shortly after salt addition.

Salinity stress tolerance in durum wheat is strongly associated with plant's ability to control Na<sup>+</sup> delivery to the shoot. Two loci, termed *Nax1* and *Nax2*, were recently identified as being critical for this process and were suggested to confer activity of *HKT1;4* and *HKT1;5* transporters from HKT gene family, respectively. At the functional level these transporters are assumed to actively retrieve Na<sup>+</sup> from the xylem thus limiting the rates of Na<sup>+</sup> transport from roots to shoots. In this work we show that *Nax* loci also affect activity and expression levels of SOS1-like Na<sup>+</sup>/H<sup>+</sup> exchanger in both root cortical and stelar tissues. Net Na<sup>+</sup> efflux measured from salt-treated stele by non-invasive ion flux measuring MIFE technique declined in the following sequence Tamaroi (parental line) > *Nax1* = *Nax2* > *Nax1:Nax2* lines. This efflux was amiloride (a known inhibitor of Na<sup>+</sup>/H<sup>+</sup> exchanger)-sensitive

and was mirrored by net  $H^+$  flux changes. SOS1 relative transcript levels were 6 to 10 fold lower in *Nax* lines compared with Tamaroi. Thus, it appears that *Nax* loci confer two highly complementary mechanisms, both contributing to reducing xylem  $Na^+$  content. One of them is enhanced retrieval of  $Na^+$  back into the root stele via HKT, and another one reduced rate of  $Na^+$  loading into the xylem via SOS1. It is suggested that such duality may play important adaptive role by providing plant with a greater versatility to respond to changing environment and control  $Na^+$  delivery to the shoot.

In conclusion, this project has found that different crop species and genotypes adopt different strategies to resist salinity stress. In barley, it was found that the predominant use of inorganic osmolytes contributes to osmotic adjustment in the shoot. While rapid  $Na^+$  delivery to the shoot seems to be an effective strategy to ensure normal shoot growth under saline condition, xylem  $Na^+$  loading should be tightly controlled and stopped once sufficient amount of  $Na^+$  was delivered to the shoot. Salt-sensitive barley cultivars failed to slow down the xylem  $Na^+$  loading once osmotic adjustment was achieved, while tolerant genotypes were efficient in controlling this process. Both HvHKT1;5 and HvSOS1 transporters were found to be involved in control of xylem  $Na^+$  loading and delivery to the shoot. The above process is also intrinsically linked with NADPH oxidase-mediated apoplastic  $H_2O_2$  production that acts upstream of xylem  $Na^+$  loading and is causally related to ROS-inducible  $Ca^{2+}$  uptake systems in the xylem parenchyma tissue. In wheat, bread and durum wheat can be differentiated by their reliance on  $Na^+$  exclusion and  $Na^+$  sequestration, respectively. The discovered role of NAX loci as controller of expression level and activity of SOS1-like transporters and existence of two highly complementary mechanisms conferring xylem  $Na^+$  loading/retrieval and reported insights into regulation of activity of membrane transporters expressed at xylem parenchyma interface open new prospects of cereal breeding for salinity tolerance.



## **Chapter 1: General introduction**

### ***Soil salinization and agricultural crop production***

Soil salinization is the accumulation of water-soluble salts in the soil to a level that impacts on agricultural production, environment health, and economic welfare (Rengasamy 2006). Soil is considered as saline if the electrical conductivity of its saturation paste extract ( $EC_e$ , equivalent to the concentration of salts in saturated soil or in a hydroponic solution) is above  $4 \text{ dS m}^{-1}$  (US Salinity Laboratory Staff, 1954). Approximately 20% the world's cultivated land, equal to 6% of the world total area, is threatened by salinity (FAO 2008). Hence, soil salinization is a global issue that affects plant growth and limits agricultural production, becoming the main challenge of the modern agriculture (Rengasamy 2006). Global food production will need to increase by 70% by 2050 (Tester and Langridge, 2010), which makes breeding salt-tolerant crop extremely urgent.

In a 2007 ranking of cereal crops in the world, barley (*Hordeum vulgare* L.) was fourth both in terms of quantity produced (136 million tons) and in area of cultivation (566,000 square kilometres or 219,000 square miles). Although *H. vulgare* is regarded as salt tolerant compared with other crops (Munns *et al.* 2006; Munns and Tester 2008), barley cultivars still experienced a 55-58% decline in a biomass at 150 mM NaCl (Greenway 1962). Wheat (*Triticum aestivum* L.) is one of the most important cereal crops worldwide that provides approximately one fifth of the total calorific input of the world's population (FAO 2010). In many wheat-growing countries of the Indian subcontinent (e.g. India and Pakistan) and Middle Eastern region (Iran, Egypt and Libya), up to 10% of the whole wheat belt is affected by salinity (Colmer *et al.* 2006). Hence, a comprehensive understanding of mechanisms of salinity tolerance in physiological and molecular level and breeding salt-tolerance genotypes in crops are critical to relief the yield penalty in barley and wheat under salinity stress.

### ***Physiological constraints imposed by salinity***

The effect of the salinity can be divided in two distinct phases through the time: the osmotic stress of the salt from the soil, and the toxicity of the salt within the plant (Munns 1993; Munns and Tester 2008). The osmotic effects of salinity stress can be observed immediately after salt application and are believed to dominate for a few weeks (Munns and James 2003), then accumulation of the toxic  $\text{Na}^+$  in plant tissues results in premature senescence and other toxicity symptoms (chlorosis, necrosis), observed first in older leaves.

During the initial phases of salinity stress, water absorption capacity of root systems decreases and water loss from leaves is accelerated due to osmotic stress of high salt accumulation in soil and plants, and therefore salinity stress is also considered as hyperosmotic stress (Munns 2005). Osmotic stress in the initial stage of salinity stress causes various physiological changes, such as nutrient imbalance, membrane depolarization, impairs the ability to detoxify reactive oxygen species (ROS) and to activate/inactivate the antioxidant enzymes, and decrease stomatal aperture and the overall ability for  $\text{CO}_2$  assimilation (Munns and Tester 2008; Rahnama *et al.* 2010). The primary impact of osmotic stress brought by salinity is the reduction of the leaf growth. A sudden increase of salt in the soil triggers leaf cell to lose water, further causing a transient decrease of cell volume and cell turgor (Passioura and Munns 2000). Organic osmolyte will be synthesised or inorganic osmolytes will be compartmentalized, for the recovery of original volume and turgor within hours. Despite this, cell elongation rates are reduced irreversibly (Passioura and Munns 2000; Fricke and Peters 2002). Consequently, this rate reduction will lead to the growth retardation, early flowering and at last, yield penalty.

$\text{Na}^+$  toxicity and ionic imbalance in the cytosol is the second major constraint brought by salinity. Because of the similarity in the physical and chemical properties between  $\text{Na}^+$  and  $\text{K}^+$ , the former could compete with  $\text{K}^+$  for the major binding sites in key metabolic processes in the cytoplasm, such as enzymatic reactions, protein synthesis and ribosome function (Marschner 1995). With over 50 cytoplasmic enzymes being activated by  $\text{K}^+$ , the disruption to metabolism is sever, both in the root and leaf tissues. High  $\text{Na}^+$  concentration in the soil will

also attenuate the uptake of other nutrients, causing further stress on plant growth. There are a few different strategies for plant to reduce cytosolic  $\text{Na}^+$  concentration. This includes: (1) exclusion of  $\text{Na}^+$  from root uptake (Gorham *et al.* 1990; Munns *et al.* 2006), (2) minimizing entry of  $\text{Na}^+$  into xylem vessels from the root symplast (Schachtman *et al.* 1992; Davenport *et al.* 2005; Garthwaite *et al.* 2005), and (3) compartmentalization of  $\text{Na}^+$  in cell vacuoles (Apse *et al.* 1999; Brini *et al.* 2007). For most plants, the movement of  $\text{Na}^+$  from the shoot to the roots in the phloem can likely recirculate only a small proportion of the  $\text{Na}^+$  that is delivered to the shoot. As such, the crucial process which determines  $\text{Na}^+$  accumulation in the shoot is primarily the processes controlling the net delivery of  $\text{Na}^+$  into the root xylem.

While a significant progress has been achieved in understanding fundamental mechanisms conferring salinity stress tolerance over the last two decades, several key issues remain unanswered:

1. How does osmotic adjustment and stomatal characteristics contribute to salinity stress tolerance in moderate salinity stress?

For plants to grow in saline soil they need to adjust osmotically to maintain a positive turgor pressure. Consequently, a cell must contain a total solute concentration greater than that of the external solution. Metabolic acclimation via the accumulation of compatible solutes is often regarded as a basic strategy for the protection and survival of plants under abiotic stress (Hanson and Hitz 1982; Sakamoto and Murata 2000; Shabala and Cuin 2006). Many plants species accumulate significant amounts of glycine betaine, proline and polyols in response to salinity (Rhodes and Hanson 1993; Bohnert *et al.* 1995; Di Martino *et al.* 2003). In addition to the conventional role of these compatible solutes in cell osmotic adjustment (Yancey *et al.* 1982; Bray 1993), they are also suggested to act as low-molecular-weight chaperones, stabilizing the photosystem II complex, protecting the structure of enzymes and proteins maintaining membrane integrity and scavenging ROS (Robinson and Jones 1986; Smirnov and Cumbe 1989; McCue and Hanson 1990; Santoro *et al.* 1992; Bohnert *et al.* 1995; Papageorgiou and Murata 1995; Shen *et al.* 1997; Hare *et al.* 1998;

Mansour 1998; Noiraud *et al.* 2001). Besides compatible solutes,  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  are the major components of the cellular osmotic potential. Accommodating high concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in tissues is generally thought to be achieved by intracellular compartmentation in the vacuole matched by *de novo* synthesis of organic solutes to adjust osmotic potential of the cytoplasm (Flowers and Colmer, 2008). Organic solutes (these include sucrose, sugar alcohols, proline and glycine betaine; Flowers and Colmer, 2008; Gil *et al.* 2013) are accumulated and most probably contribute to osmotic adjustment in the cytoplasmic compartments of vacuolated cells (Flowers *et al.* 1977; Wyn Jones *et al.* 1977; Greenway and Munns 1980) rather than the whole cell. From the energetic point of view, cellular osmotic adjustment is more efficiently achieved by the use of ions than organic solutes (Greenway and Munns 1983; Yeo, 1983), the synthesis of which would divert C and N from the supply of assimilates for growth processes (e.g. for N in *Spartina alterniflora*; Colmer *et al.* 1996). As large amounts of energy would be needed to synthesize these organic compounds in sufficient quantities to adjust the whole volume of cells, it follows that evolutionary pressure has resulted in plants using  $\text{Na}^+$  and  $\text{Cl}^-$  as osmotica in vacuoles while preventing these ions rising to toxic concentrations in the cytoplasm (Flowers *et al.* 2010). Entry of these ions into some critical cells and tissues, such as meristems and very young growing cells, might also be restricted to keep concentrations relatively low (Greenway and Munns, 1980; Flowers *et al.* 2010).

Osmotic component of salinity stress has a profound effect on stomatal characteristics, causing reduction in the transpiration rates and an ultimate stomatal closure (Brugnoli and Lauteri 1991). Stomatal closure results in stomatal limitations to photosynthesis through reduced  $\text{CO}_2$  availability, leading to  $\text{CO}_2$  assimilation and the detrimental processes of photorespiration (Noctor *et al.* 2002; Geissler *et al.* 2008). A positive correlation has been found between  $g_s$  and yield in barley and other species under saline conditions (Seemann and Critchley 1985; Brugnoli and Lauteri 1991; Jiang *et al.* 2006). Both stomata aperture and stomata density may affect the changes in  $g_s$  under salinity. It was argued that changes in the stomata density may represent a fundamental

mechanism by which plant can optimise water use-efficiency under high saline conditions (Shabala *et al.* 2012; Shabala *et al.* 2013), as reducing the density of stomata (and hence the number of cuticular pores associated with them) would be beneficial to osmotically stressed plants. However, to the best of our knowledge no study has linked stress-induced changes in stomatal density with salinity stress tolerance in glycophyte crop species using sufficient large number of accessions. Is it good to plant to have fewer stomata, to control transpiration? Or is it better to have many partially closed stomata? Also, salinity-brought osmotic stress requires osmotic adjustment, which can be achieved by either an increase in compatible solute production or increased uptake of inorganic ions ( $\text{Na}^+$ ,  $\text{K}^+$  or  $\text{Cl}^-$ ; Munns and Tester 2008; Quintero *et al.* 2008; Mian *et al.* 2011). What is better for plants: to exclude  $\text{Na}^+$  from uptake, or use it as a cheap osmoticum? Most of previous studies addressed this question for plants grown under extremely severe saline conditions (300 mM NaCl or even more; Garthwaite *et al.* 2005; Islam *et al.* 2007; Fatehi *et al.* 2012). While in the real field, plants may be exposed to more mild salinity (Rengassamy 2002, 2006), and thus, may require different mechanisms to cope with salinity tolerance. So, is there any difference in the optimal strategy to deal with this issue?

2. What is the contribution rate of each of various physiological indices for salinity tolerance in wheat?

Several physiological and agronomical characteristics have been used as a proxy for salinity stress tolerance. This include relative water content, germination rate (Mano and Takeda 1997; Tajbakhsh *et al.* 2006), dry and wet weight of roots and shoots (Chen *et al.* 2005; Siahshar *et al.* 2009), leaf injury and reduction of  $\text{CO}_2$  assimilation (Munns *et al.* 2010), loss of chlorophyll and damage to the photosynthetic apparatus (Krishnaraj *et al.* 1993),  $\text{Na}^+$  exclusion from the shoot (Garcia *et al.* 1995),  $\text{K}^+/\text{Na}^+$  discrimination (Asch *et al.* 2000),  $\text{Cl}^-$  exclusion (Rogers and Noble 1992) and  $\text{K}^+$  flux under salinity stress (Chen *et al.* 2005). Each of these may be controlled by a large number of genes. Accordingly, most researchers agree that the best way to proceed with breeding would be via pyramiding different useful physiological traits. However, in spite of substantial efforts, the outcomes are still disappointingly poor due to the

physiological and genetic complexity of this trait, the lack of reliable screening tools and, most importantly, the lack of a comprehensive understanding of the mechanisms behind salinity tolerance. Moreover, most of the previous studies on salt tolerance mechanisms are based on a rather limited number of varieties (Brugnoli and Lauteri 1991; Chen *et al.* 2007). The results can be affected by both plant's tolerance to salt stress and genetic background of the selected varieties.

3. What is the physiological and molecular mechanisms mediating xylem  $\text{Na}^+$  loading in wheat in context of salinity stress?

Among the multiple physiological mechanisms conferring salinity tolerance, control of xylem ion loading and regulation of ionic exchange at the xylem-parenchyma boundary have often been named as central to salinity tolerance (Munns and Tester 2008; Tester and Davenport 2003). With the application of molecular biology techniques, several candidate genes playing an important role in controlling xylem ion relations were characterized. Amongst these are  $\text{Na}^+/\text{H}^+$  antiporter (SOS1; Shi *et al.* 2002; Shi *et al.* 2003; Feki *et al.* 2011; Feki *et al.* 2014), high-affinity  $\text{Na}^+/\text{K}^+$ -permeable transporter (HKT; reviewed by Horie *et al.* 2009), chloride cation co-transporter (CCC; Colmenero-Flores *et al.* 2007), and Shaker-like outward channel (SKOR; Gaymard *et al.* 1998). All these transporters were found to potentially mediate xylem  $\text{K}^+$  and  $\text{Na}^+$  loading and retrieval.

Even though the molecular identity of candidate transporters potentially mediating xylem  $\text{Na}^+$  and  $\text{K}^+$  loading has been characterised, the process of their regulation is much less understood. The ABA induced inhibition of channel-mediated efflux of  $\text{K}^+$  into xylem has been well documented (Cram and Pitman 1972; Roberts 1998; Gilliham 2002); however, little is known about how ABA controls xylem  $\text{Na}^+$  loading. One interesting observation is that ABA application stimulated  $\text{H}^+$  extrusion into xylem, so this could potentially enhance active xylem loading via stimulation of  $\text{Na}^+/\text{H}^+$  antiporter activity in the plasma membrane of xylem parenchyma cells (Clarkson and Hanson 1986; Hall *et al.* 2006). Besides ABA,  $\text{H}_2\text{O}_2$  could be another candidate regulating xylem ion loading. However, information on this matter is also scarce. Meanwhile, ROS

were shown to activate Na<sup>+</sup>-permeable non-selective cation channels (NSCCs) in both roots (Demidchik and Tester 2002; Demidchik *et al.* 2003; Carvalho *et al.* 2010; Zepeda-Jazo *et al.* 2011) and leaves (Pei *et al.* 2000; Kwak *et al.* 2003). Activation of the K<sup>+</sup>-permeable outward rectifying GORK channel by OH<sup>-</sup> was observed in protoplasts isolated from *Arabidopsis* roots (Demidchik *et al.* 2010). Revealing the possible role of ABA and H<sub>2</sub>O<sub>2</sub> as candidate second messengers regulating xylem ion loading was another aim of this study.

Little is known about the molecular nature of the transporters moving ions into/out of the xylem, with even the energetics of the process being uncertain (Tester and Davenport 2003). This profound lack of knowledge is remarkable because these processes are clearly very important. Is there any difference in the kinetics of xylem ion loading under salinity stress between salt-tolerant and salt-sensitive genotypes? How is the loading controlled at physiological level? What is the molecular identity of the transport systems behind this process, and to what extent changes in their expression pattern contribute to this process?

4. What is the impact of *Nax* (Na<sup>+</sup> exclusion) loci on the expression and activity of SOS-like Na<sup>+</sup>/H<sup>+</sup> exchanger in wheat?

Among wheat varieties, durum (pasta) wheat (*Triticum turgidum* L. subsp. *durum*) is generally less tolerant to salt stress than bread wheat (*Triticum aestivum*) (Munns *et al.* 2006; Munns and Tester 2008), mainly because of high rates of Na<sup>+</sup> accumulation and poor K<sup>+</sup>/Na<sup>+</sup> discrimination (Chen *et al.* 2007; Gorham *et al.* 1990; Munns and James 2003). To improve salt tolerance of durum wheat, two loci that conferred reduced Na<sup>+</sup> accumulation in the shoot, termed *Nax1* and *Nax2*, were found in an unusual genotype named Line 149 (Munns *et al.* 2003). Lines containing either *Nax1* or *Nax2* loci were observed having lower rates of Na<sup>+</sup> transport from roots to shoots, due to the lower rate of net Na<sup>+</sup> loading into the xylem (James *et al.* 2006). *Nax1* was indentified by fine mapping as a Na<sup>+</sup> transporter of the *HKT* gene family *TmHKT1;4* (Huang *et al.* 2006), while *Nax2* was located on chromosome 5A and identified as *TmHKT1;5* (Byrt *et al.* 2007). It was localised on the plasma membrane of cells surrounding the xylem, and was found to confer a yield benefit of 25% on saline soil (Munns *et al.* 2012).

AtSOS1 has been identified as Na<sup>+</sup>/H<sup>+</sup> antiporters localised in epidermal cells at the root tip where they actively extrude Na<sup>+</sup> from cytosol into rhizosphere (Shi *et al.* 2002). *SOS1* expression signal was also strong in the parenchyma root cells at the xylem/symplast boundary (Shi *et al.* 2002), where it may mediate xylem Na<sup>+</sup> loading and delivery to the shoot. Overexpression of SOS1 in transgenic *Arabidopsis* has been shown to improve salt tolerance (Shi *et al.* 2003). Feki *et al.* have identified the *TdSOS1* gene from durum wheat, and the heterologous expression of *TdSOS1* in a yeast strain lacking endogenous Na<sup>+</sup> efflux proteins showed complementation involving cation efflux (Feki *et al.* 2011).

However, little is known about the impact of *Nax* loci on activity and expression levels of SOS1-like Na<sup>+</sup>/H<sup>+</sup> exchanger in wheat, and what the relationship between *SOS1* gene expression pattern and overall performance of *Nax* lines under salinity stress.

### ***Objectives and research aims***

Based on the aforementioned unknown questions, there are four main objectives in this research:

- To explore the relationship between osmotic adjustment and stomatal characteristics with salinity stress tolerances in contrasting barley accessions

In this part, the aim was to fill in the gap in our knowledge and advance our understanding of the mechanisms underlying salinity stress tolerance in plants. This was achieved by physiological assessment of a broad range of barley genotypes and correlating the overall salinity tolerance in this species with changes in stomatal characteristics and the contribution of inorganic osmolytes towards to the shoot osmotic adjustment. These experiments were conducted at moderate salinity stress.

- To evaluate predictive values of various physiological indices for salinity stress tolerance in bread and durum wheat



In this part, the following questions were addressed by using a large number of durum and bread wheat accessions: which physiological traits make the biggest contribution towards the overall salinity tolerance (measured as relative yield under salt stress), and which parameters can be omitted as having low predictive value?

- To investigate the physiological and molecular mechanisms mediating xylem  $\text{Na}^+$  loading in wheat in context of salinity stress

The main objective of this part was to answer the following questions: is there a difference in the kinetics of xylem ion loading under salinity stress between salt-tolerant and salt-sensitive genotypes? How this loading is controlled, at physiological level? What is the molecular identity of the transport systems behind this process, and to what extent changes in their expression pattern contribute to this process? The overall goal of this work was to offer a comprehensive insight into mechanisms of ion loading/unloading in xylem in the context of salinity stress.

- To evaluate the impact of *Nax* ( $\text{Na}^+$  exclusion) loci on the expression and activity of SOS-like  $\text{Na}^+/\text{H}^+$  exchanger in wheat

In this part, we used *Nax1* and *Nax2* durum wheat lines to provide the supporting evidence for the role of SOS1-mediated  $\text{Na}^+$  loading into the xylem in this species. The aim of this part was to test the hypothesis that reduced  $\text{Na}^+$  accumulation in the shoot of *Nax* lines could be conferred not only by higher  $\text{Na}^+$  retrieval from but also by reduced  $\text{Na}^+$  loading into the xylem.

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## ***Chapter 2: Linking osmotic adjustment and stomatal characteristics with salinity stress tolerance in contrasting barley accessions<sup>1</sup>***

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### **Abstract**

Salinity tolerance is a complex trait, both physiologically and genetically, and the issue of which mechanism or trait has bigger contribution towards the overall plant performance is still hotly discussed in the literature. In this work, a broad range of barley (*Hordeum vulgare* and *Hordeum spontaneum*) genotypes contrasting in salinity stress tolerance were used to investigate the causal link between plant stomatal characteristics, tissue ion relations, and salinity tolerance. In total, 46 genotypes (including two wild barleys) were grown under glasshouse conditions and exposed to moderate salinity stress (200 mM NaCl) for 5 weeks. It was found that the overall salinity tolerance correlated positively with stomata density, leaf  $K^+$  concentration and the relative contribution of inorganic ions towards osmotic adjustment in the shoot. At the same time, no correlation between salinity tolerance and stomatal conductance or leaf  $Na^+$  content in shoot was found. Taken together, these results indicate the importance of increasing stomata density as an adaptive tool to optimise efficiency of  $CO_2$  assimilation under moderate saline conditions, as well as benefits of the predominant use of inorganic osmolytes for osmotic adjustment, in barley. Another interesting finding was that wild barleys showed rather different strategies dealing with salinity, as compared with cultivated varieties.

**Key words:** stomatal conductance; stomata density; sodium; potassium; organic osmolytes; inorganic osmolytes

### **Introduction**

Soil salinization is a global issue that affects plant growth and limits agricultural production. Approximately 20% of the world's cultivated land, which accounts for over 6% of the world total area, is threatened by salinity (FAO 2008). Soil salinization has become one of the main challenges of the modern agriculture,

calling for an urgent need to develop salt-tolerant genotypes (Flowers and Colmer 2008; Shabala 2013).

The classical view is that salinity affects plant performance via osmotic and ion-specific mechanisms (Greenway 1963; Greenway and Munns 1980; Munns and Tester 2008) and, therefore, occurs in two distinct phases. The osmotic effects of salinity stress can be observed immediately after salt application and are believed to dominate for a few weeks (Munns 2002). The following accumulation of the toxic  $\text{Na}^+$  in plant tissues then results in a premature senescence and other toxicity symptoms (chlorosis, necrosis), observed first in older leaves. The idea of two mechanisms operating at different timescales is an attractive one and was used to separate osmotic and ionic components of salt stress on numerous occasions (Munns *et al.* 1995; Yeo *et al.* 1991; Rajendran *et al.* 2009). However, the boundary between two components seems to be rather diffuse. Salinity-induced programmed cell death (PCD) was observed in salt-stressed roots within few hours (Huh *et al.* 2002; Li *et al.* 2007a; Li *et al.* 2007b), and three days of 80 mM NaCl treatment resulted in a complete loss of viability in most cells in the elongation zone of pea roots (Bose *et al.* 2014b). Hence, it appears that the relative contribution of each of the above mechanisms, as well as their separation based on a timescale of operation, is highly tissue specific and strongly dependent on a severity of the salt stress. When salt stress is mild then toxicity can be avoided by efficient  $\text{Na}^+$  exclusion (Liu and Zhu 1997; Zhu *et al.* 1998; Apse *et al.* 1999) or compartmentalization in the vacuole (Asch *et al.* 2000; Zhang and Blumwald 2001; Zhang *et al.* 2001; Tester and Davenport 2003). In this case, osmotic component is most likely to dominate (Flowers and Colmer 2008; Mian *et al.* 2011). When stress is more severe and plants cannot avoid NaCl-induced accumulation of reactive oxygen species (ROS) in their tissues, toxicity component dominates and cells are killed (Ding *et al.* 2010; Bose *et al.* 2014a).

Osmotic component of salt stress has a profound effect on stomatal conductance,  $g_s$  (Seemann and Critchley 1985; Jiang *et al.* 2006). Previous attempts to correlate salinity tolerance in barley with  $g_s$  have found positive correlations

between  $g_s$  and yield under saline conditions (Jiang *et al.* 2006); the same trend was observed in some other species (Seemann and Critchley 1985; Brugnoli and Lauteri 1991). However, changes in  $g_s$  can be due to both changes in aperture of the stomata pore as well as altered stomata density (SD). It was argued that changes in the stomata density may represent a fundamental mechanism by which plant can optimise water use-efficiency under high saline conditions (Shabala *et al.* 2012; Shabala *et al.* 2013), as reducing the density of stomata (and hence the number of cuticular pores associated with them) would be beneficial to osmotically stressed plants. However, to the best of our knowledge no study has linked stress-induced changes in SD with salinity stress tolerance in glycophyte crop species using sufficiently large number of accessions. Is this trait important for salinity tolerance? Is it beneficial for a broad range of salinity concentrations? Should it be targeted in breeding programs?

Salinity-brought osmotic stress requires osmotic adjustment, which can be achieved by either an increase in compatible solute production or increased uptake of inorganic ions ( $\text{Na}^+$ ,  $\text{K}^+$  or  $\text{Cl}^-$ ) (Munns and Tester 2008; Quintero *et al.* 2008; Mian *et al.* 2011). A collection of 69 barley genotypes grown at 150 mM NaCl in hydroponics was screened for their salt tolerance and shoot  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{K}^+$  content (Tavakkoli *et al.* 2012) but failed to find any correlations between salt tolerance and tissue content of any of above ions. At the same time, some early studies on barley reported a negative correlation between the amount of compatible solutes levels and salinity tolerance (Chen *et al.* 2007), suggesting that accumulation of compatible solutes in this species was likely to be a symptom of stress rather than a beneficial trait that contributes to salinity stress tolerance. However, with only four contrasting genotypes used that conclusion needed to be validated in a large-scale experiments, to demonstrate the relationship between compatible solute levels and salinity tolerance. So, the question remains if it is good or bad for barley to rely on compatible solutes for its osmotic adjustment under saline conditions.

Also, most previous works on barley have been conducted under severe conditions (300 mM  $\text{Na}^+$  or more) (Garthwaite *et al.* 2005; Islam *et al.* 2007;

Fatehi *et al.* 2012). While this approach was highly useful for the accurate phenotyping and separating contrasting varieties (Chen *et al.* 2005; Islam *et al.* 2007), plants grown in the field may be exposed to more mild salinity (Rengasamy 2002, 2006), and thus might require different mechanisms to deal with salinity tolerance. Hence, the best performing varieties selected under severe salt conditions may be not necessarily doing their best when plants are subject to moderate and extreme saline environments (El-Hendawy *et al.* 2009).

The aim of this study was to fill in the above gaps in our knowledge and advance our understanding of the mechanisms underlying salinity stress tolerance in plants. This was achieved by physiological and agronomical assessment of a broad range of barley accessions and associating the overall salinity tolerance in this species with changes in stomatal characteristics and the contribution of inorganic osmolytes towards the shoot osmotic adjustment. These experiments were conducted at moderate salinity stress, to mimic “real field” conditions. Overall, our results indicate the importance of increasing stomata density as an adaptive tool to optimise CO<sub>2</sub> assimilation efficiency, as well as benefits of the predominant use of inorganic osmolytes for osmotic adjustment under moderate saline conditions in barley.

## **Material and methods**

### ***Plant material and growth condition***

Barley (*Hordeum vulgare* and *Hordeum spontaneum*) seeds were obtained from the Australian Winter Cereal Collection and from the barley genotype collection of Zhejiang and Yangzhou Universities in China. 46 barley genotypes were grown in a glasshouse at the Mount Pleasant Laboratory facilities in Launceston. The experiment was conducted in 2011 (June to November) and the daily temperatures were 24°C (in the day) and 16°C (at night). Plants were grown in a 40-L poly (vinyl) chloride (PVC) container (two genotypes per container) filled with the fertilised standard potting mix (see Chen *et al.* 2007 for details). 30 seeds were planted for each genotype. The emerged seedlings (one week after sowing) were treated with 200 mM NaCl for 5 weeks. Plants were watered with



excessive amounts of salt solution several times per day (run for waste). As a result, the concentration of NaCl in the potting mix was stable and matched that of the irrigation solution (200 mM NaCl).

#### *Stomatal conductance and density measurements*

Stomatal conductance was measured in the youngest fully-expanded leaf using leaf porometer (model SC-1, Decagon, Australia). Measurements were taken between 9.30am-11.30am and 14.30pm-16.30pm, under glasshouse conditions, during the sunny days (1000-1200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Seven replicates for each cultivar were measured, for each of salt-treated and control treatments. During data analysis, the maximum and minimum machine reading values were removed and the rest five values were averaged.

To acquire stomata density, abaxial barley leaf surface was coated with clear nail varnish, and then the dried layer of nail varnish was peeled off using tweezers and stick onto a glass slide. The number of stomata was counted for each field of view. For each of genotype/treatment, 3 imprints from 3 biological replicates were analysed.

#### *Leaf elemental content and osmolality (Osm)*

The youngest fully-expanded leaf was harvested (6 replicates for each cultivar for both salt-treated and control plants) after five weeks of NaCl treatment. Leaves were hand squeezed in the Eppendorf tubes to extract the sap (as described in Cuin *et al.* 2009), and then the samples were centrifuged at 8,000 rpm for 10 minutes. 20  $\mu\text{l}$  of the collected supernatant was measured for its osmolality using a vapour pressure osmometer (Vapro; WescorInc, Logan, UT, USA). In addition, 50  $\mu\text{l}$  of the collected supernatant was mixed with 5 ml distilled water and the mixture was assessed in a flame photometer (Corning 410C, Essex, UK) to quantify  $\text{K}^+$  and  $\text{Na}^+$  concentration in the leaf sap.

*Scoring for salinity stress tolerance*

Barley varieties were grown in 4L PVC containers as described earlier. Six seeds of each cultivar were sown in each pot and growth under condition described above. One week after germination, 320 mM NaCl was gradually added to the irrigation solution (40 mM increments per day). Salinity treatment continued for 4 weeks, and then the degree of leaf injury and the number of surviving plants were recorded and scored. The extent of leaf injury was then ranked on 0 to 10 scale (0 – no visual symptoms; 10 – dead plants; see Fig. 1 for details). The reasons for using higher (320 mM but not 200 mM) salinity levels for plant screening were two fold. First, such an approach allowed the qualitative (e.g. alive vs dead) evaluation and, hence, was more conclusive. Second, tolerance to more severe 320 mM NaCl stress require the presence of all component of salt stress tolerance (e.g. both osmo- and tissue-tolerance) and, thus, was better suited for estimation of the overall salinity stress tolerance, while handling more modest 200 mM NaCl treatment might rely predominantly on plant ability to deal with osmotic stress.

*Data analysis*

Data were analysed using IBM SPSS Statistics 21 for Windows. All results are given as means  $\pm$  SE. Then one-way ANOVA was used to calculate the significance of differences between the results. Different low-case letters in each panel of the figures indicate significance at  $P < 0.05$  level.

**Results**

Forty six barley varieties were grown under glasshouse conditions under 320 mM NaCl irrigation for 4 weeks and scored according to the degree of leaf injury (Fig. 1). Based on this, these genotypes were clustered into 4 groups: highly tolerant (injury score index  $\leq 0.5$ ), tolerant (1 to 1.75), moderately tolerant (2 to 2.75) and sensitive (3 to 9.5) groups (Table 1). There are only two varieties in

highly tolerant group, CPI 11284-48 and SYR01, which are also the only two wild barleys among all 46 barley varieties. The tolerant group includes a few varieties frequently used in previous studies such as TX9425, CM72, Numar, and ZUG293 (Chen *et al.* 2007; Chen *et al.* 2008; Shabala *et al.* 2010). The ‘core’ sensitive cultivars (Franklin, Gairdner, ZUG403, Naso Nijo, and Unicorn) were all located in the sensitive group, indicating the reliance of using leaf injury as the indicator for salt tolerance. A strong correlation ( $R^2 = 0.50$ ) was observed between score index in this study and grain yield data reported in previous studies (Chen *et al.* 2007; Fig. 2), using 12 “shared” cultivars (TX9425, CM72, YWHKSL, YYXT, Numar, ZUG293, Lixi 143, YSM 3, Hu 93-045, Aizao 3, Gairdner, YPSLDM and ZUG403), and a significant ( $P < 0.01$ ) negative correlation was reported between relative FW and leaf injury index using the same set of 46 genotypes (Wu *et al.* 2014), suggesting that either of these indices may be used as an indicator of salinity tolerance.

With an exception of Numar and Unicorn, a significant reduction was found in the stomatal conductance of the remaining 44 barley cultivars under moderate salinity (200 mM NaCl for 35 days) compared to stomatal conductance in control plants (Fig. 3). As changes in  $g_s$  could be due to the changes in both stomata aperture and stomata density, the stomata density was determined and analysed here (Fig. 4). Generally, stomata density increased after intermediate saline treatment (Fig. 4), with an exception of Keel and Kinu Nijo 6. In contrast, salinity stress caused a significant decrease in stomatal conductance. However, no significant (at  $P < 0.05$ ) correlation was observed between these two characteristics (Table 2). The mean  $g_s$  and SD values were then pooled within each of four groups (Fig. 5). No significant (at  $P < 0.05$ ) difference of stomatal conductance and density were observed between the groups (Fig. 5). At the same time, an interesting trend was observed for SD in control, with SD being ranked in the order  $W < T < M = S$  (W – wild barley; T – tolerant genotypes; M – moderately sensitive genotypes; S – sensitive genotypes). A larger sample size for each group could potentially make this trend significant.

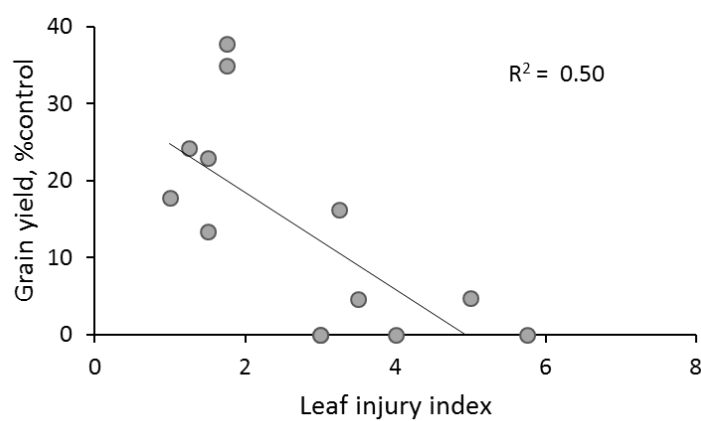


Shoot sap osmolality increased in salt-grown plants (Fig. 6). The lowest increase (19%) was observed in Kinu Nijo 6, while the highest (190%) was measured in YSM 1. No distinct patterns among different groups were found. A significant correlation between relative osmolality under saline conditions and relative leaf  $\text{Na}^+$  content under saline conditions was observed in Table 2 ( $R = 0.69$ ,  $P < 0.001$ ), indicating that the increase of shoot sap osmolality might be due to the leaf  $\text{Na}^+$  accumulation after salt application.

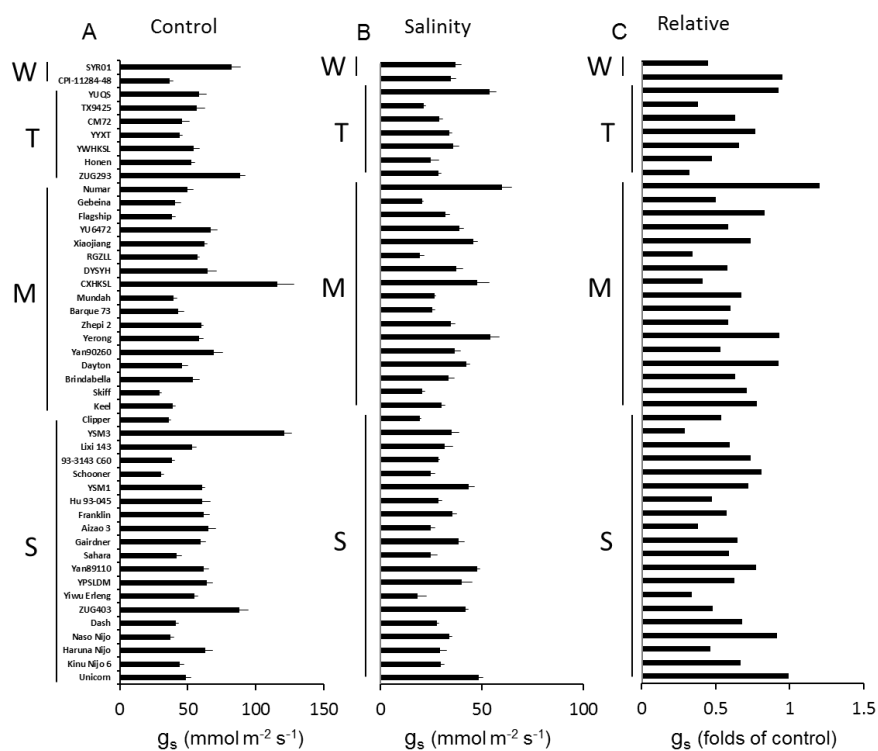
**Table 1** Genotypes ranking according to salinity tolerance measured by score index of leaf injury (0 – no visual symptoms; 10 – dead plants). Four major groups are distinguished: W – wild barley; T – tolerant genotypes; M – moderately sensitive genotypes; S – sensitive genotypes.

Genotypes	Score	Genotypes	Score	Genotypes	Score
<i>W group</i>		<i>M group</i>		<i>S group</i>	
SYR01	0.25	DYSYH	2.00	Schooner	3.00
CPI-11284-48	0.50	RGZLL	2.00	93-3143	3.00
		Xiaojiang	2.00	Lixi 143	3.00
<i>T group</i>		YU6472	2.00	YSM 3	3.00
TX9425	1.00	Barque 73	2.25	Franklin	3.25
YUQS	1.00	Mundah	2.25	Hu 93-045	3.25

CM72	1.25	CXHKSL	2.25	YSM 1	3.25
Honen	1.50	Keel	2.50	Aizao 3	3.50
YWHKSL	1.50	Skiff	2.50	Sahara	4.00
YYXT	1.50	Brindabella	2.50	Gairdner	4.00
Flagship	1.75	Dayton	2.50	Yan89110	4.50
Gebeina	1.75	Yan90260	2.50	Yiwu Erleng	5.00
Numar	1.75	Yerong	2.50	YPSLDM	5.00
ZUG293	1.75	Zhepi 2	2.50	ZUG403	5.75
		Clipper	2.75	Dash	6.00



**Fig. 2.** Correlation between salt injury index and relative grain yield of 12 “core” barley varieties (TX9425, CM72, YWHKSL, YYXT, Numar, ZUG293, Lixi 143, YSM3, Hu 93-045, Aizao 3, Gairdner, YPSLDM and ZUG403). Yield data is taken from Chen *et al.* (2007).

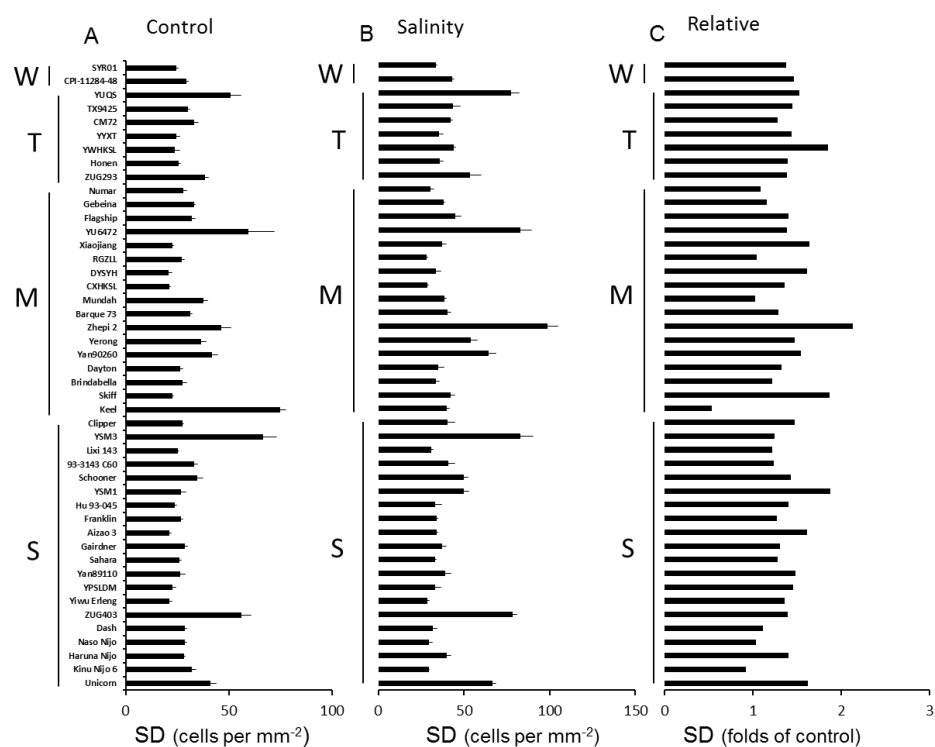


**Fig. 3.** Stomatal conductance ( $g_s$ ;  $\text{mmol m}^{-2} \text{s}^{-1}$ ) of 46 barley varieties grown under control and salt stress (200 mM NaCl for 35 days) conditions. Mean  $\pm$  SE ( $n = 6$ ). A – Control; B – salinity; C – relative (% control).

**Table 2** Correlations between agronomic and physiological characteristics under salinity stress

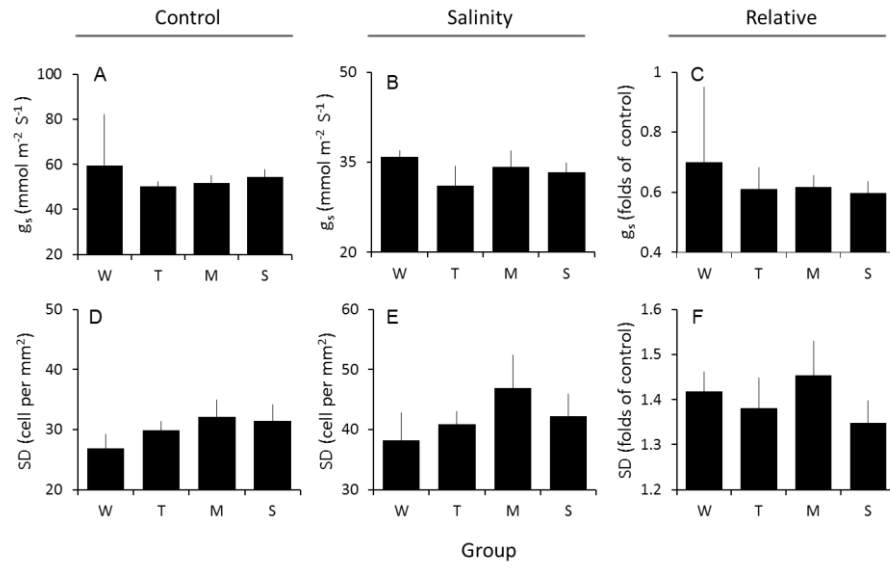
	Score	g <sub>s</sub> (C)	g <sub>s</sub> (S)	g <sub>s</sub> (R)	g <sub>s</sub> (D)	SD(C)	SD(S)	SD(R)	SD(D)	Osm(C)	Osm(S)	Osm(R)	Osm(D)	Leaf Na(C)	Leaf Na(S)	Leaf Na(R)	Leaf Na(D)	Leaf K(C)	Leaf K(S)	Leaf K(R)	Leaf K(D)	K/Na-leaf(S)
Score	1.00																					
g <sub>s</sub> (C)	-0.06	1.00																				
g <sub>s</sub> (S)	0.03	0.34	1.00																			
g <sub>s</sub> (R)	0.08	-0.53	0.57	1.00																		
g <sub>s</sub> (D)	-0.08	0.86	-0.19	-0.86	1.00																	
SD(C)	0.01	0.22	0.15	0.02	0.15	1.00																
SD(S)	-0.04	<b>0.32</b>	0.27	-0.01	0.19	0.73	1.00															
SD(R)	-0.14	0.14	0.18	-0.03	0.05	-0.26	0.41	1.00														
SD(D)	-0.06	0.23	0.24	-0.04	0.11	0.00	0.69	0.88	1.00													
Osm(C)	0.22	<b>0.30</b>	0.09	-0.15	0.27	<b>0.50</b>	<b>0.45</b>	-0.18	0.12	1.00												
Osm(S)	0.18	<b>0.41</b>	<b>0.33</b>	-0.05	0.24	<b>0.63</b>	<b>0.78</b>	0.27	<b>0.46</b>	0.57	1.00											
Osm(R)	0.02	0.09	0.26	0.11	-0.04	0.10	<b>0.33</b>	<b>0.50</b>	<b>0.37</b>	-0.45	0.47	1.00										
Osm(D)	0.05	0.25	<b>0.33</b>	0.06	0.08	<b>0.36</b>	<b>0.59</b>	<b>0.47</b>	<b>0.47</b>	-0.10	0.76	0.92	1.00									
Leaf Na(C)	-0.06	<b>-0.38</b>	<b>-0.36</b>	0.06	-0.20	<b>-0.30</b>	<b>-0.45</b>	-0.28	<b>-0.34</b>	-0.21	<b>-0.66</b>	<b>-0.50</b>	<b>-0.63</b>	1.00								
Leaf Na(S)	0.00	<b>-0.40</b>	-0.12	0.22	<b>-0.35</b>	-0.27	<b>-0.46</b>	-0.21	<b>-0.38</b>	<b>-0.44</b>	<b>-0.34</b>	0.14	-0.07	0.39	1.00							
Leaf Na(R)	-0.02	0.14	0.29	0.06	-0.01	0.06	0.17	0.28	0.19	-0.19	<b>0.38</b>	<b>0.69</b>	<b>0.61</b>	-0.62	0.26	1.00						
Leaf Na(D)	0.03	<b>-0.31</b>	-0.01	0.22	<b>-0.32</b>	-0.19	<b>-0.35</b>	-0.13	<b>-0.30</b>	<b>-0.40</b>	-0.15	<b>0.31</b>	0.14	0.10	0.95	0.49	1.00					
Leaf K(C)	0.07	0.22	0.09	-0.05	0.18	<b>0.35</b>	<b>0.30</b>	-0.06	0.06	<b>0.50</b>	<b>0.52</b>	0.01	0.23	<b>-0.34</b>	-0.17	0.08	-0.07	1.00				
Leaf K(S)	-0.28	0.06	0.00	-0.13	0.06	-0.19	-0.09	0.22	0.07	-0.12	0.09	0.26	0.21	-0.28	-0.15	0.25	-0.07	0.25	1.00			
Leaf K(R)	<b>-0.31</b>	-0.03	-0.03	-0.10	-0.01	<b>-0.33</b>	-0.20	0.26	0.06	<b>-0.37</b>	-0.12	0.29	0.14	-0.16	-0.09	0.24	-0.05	-0.20	0.90	1.00		
Leaf K(D)	<b>0.30</b>	0.10	0.07	0.09	0.07	<b>0.42</b>	0.29	-0.24	-0.02	<b>0.47</b>	0.28	-0.22	-0.03	0.02	0.02	-0.17	0.02	0.47	-0.73	-0.95	1.00	
K/Na-leaf (S)	-0.14	<b>0.32</b>	0.01	-0.28	<b>0.32</b>	0.24	<b>0.50</b>	<b>0.36</b>	<b>0.47</b>	<b>0.35</b>	<b>0.40</b>	0.04	0.21	<b>-0.42</b>	<b>-0.79</b>	-0.09	<b>-0.72</b>	<b>0.34</b>	<b>0.52</b>	<b>0.38</b>	-0.23	1.00

g<sub>s</sub>(stomatal conductance); SD (stomata density); Osm (osmolality); C (control); S (salinity); R (relative); D (difference). Bold numbers mean  $P < 0.05$ .

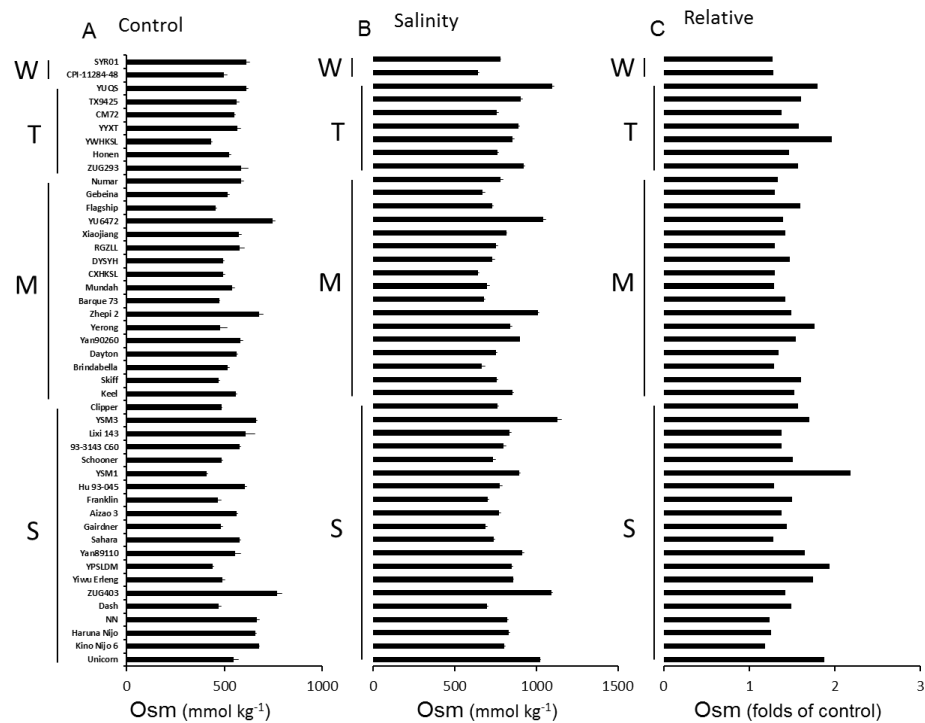


**Fig. 4.** Stomata density (SD; cells per mm<sup>-2</sup>) of 46 barley varieties grown under control and salt stress (200 mM NaCl for 35 days) conditions. Mean  $\pm$  SE (n = 6). A – Control; B – salinity; C – relative (% control).





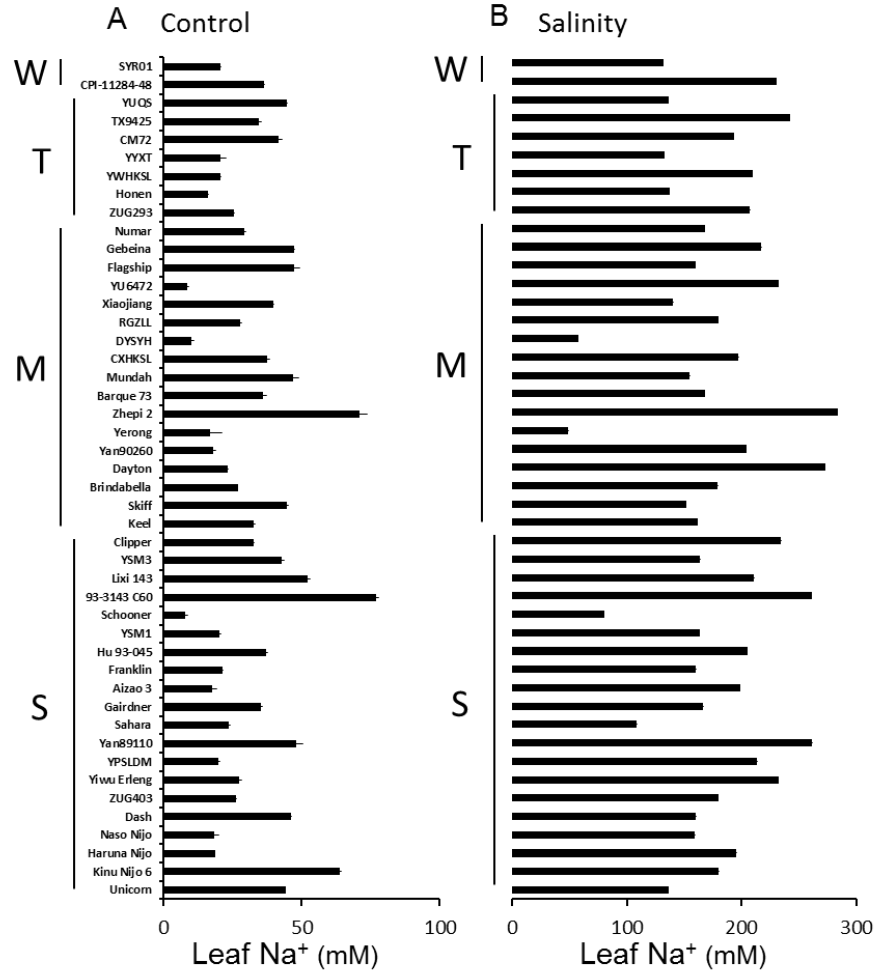
**Fig. 5.** Mean stomatal characteristics (A-C, stomatal conductance; D-E, – stomata density) for plants clustered according to their salinity stress tolerance. Cluster 1 = wild barley (W; scoring index < 1); Cluster 2 = tolerant (T; scoring index 1 – 2); Cluster 3 = moderately tolerant (M; scoring index 2 – 3); Cluster 4 = sensitive (S; scoring index > 3). Data is mean  $\pm$  SE (n = 6). A, D – control; B, E – salinity; C, F – relative (% control).



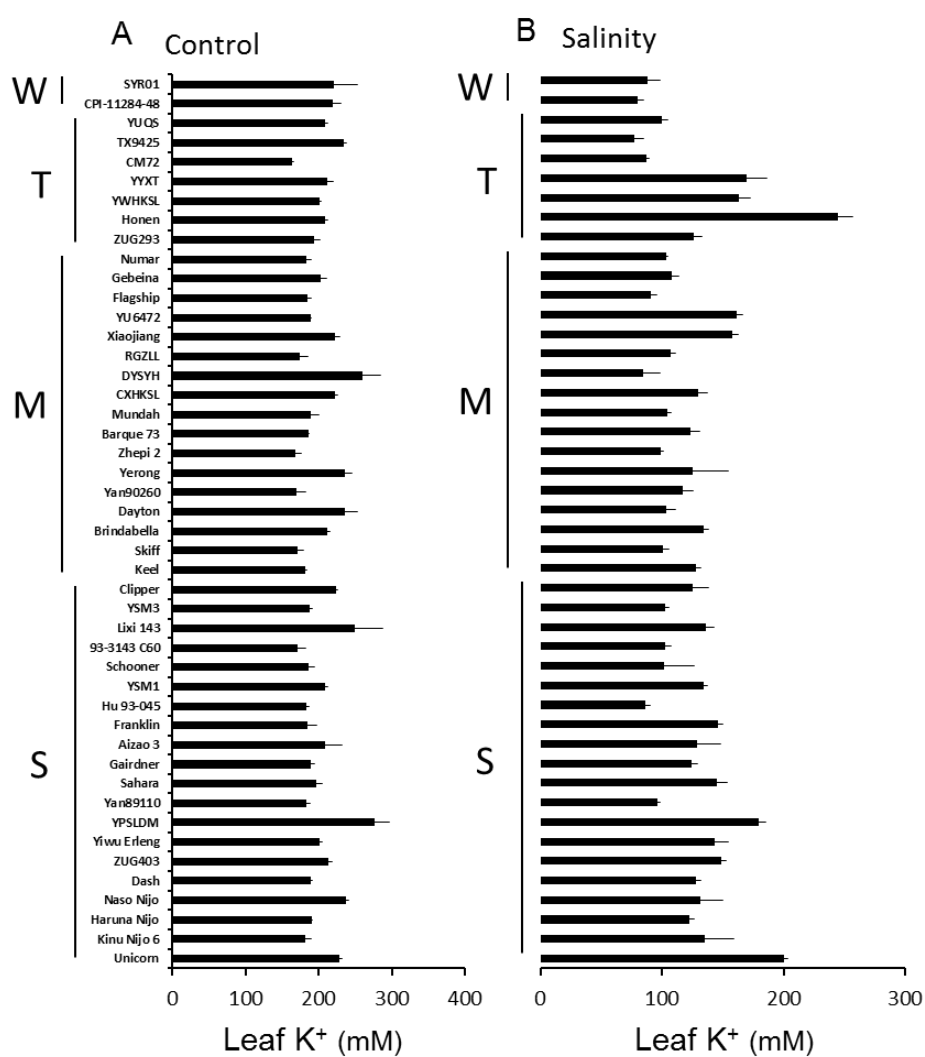
**Fig. 6.** Shoot sap osmolality (Osm; mmol kg<sup>-1</sup>) of 46 barley varieties grown under control and salt stress (200 mM NaCl for 35 days) conditions. Mean  $\pm$  SE (n = 6). A – Control; B – salinity; C – relative (% control).

Leaf Na<sup>+</sup> content in salinity-affected plants increased dramatically (Fig. 6); however, there is no significant correlation between leaf Na<sup>+</sup> content and leaf injury index among all the 46 barley genotypes under either saline or non-saline conditions (Table 2). With an exception of Yiwu Erleng and YSM 1, there was a significant reduction in sap shoot K<sup>+</sup> concentration in saline-grown plants (Fig. 7). Relative leaf K<sup>+</sup> concentration under salinity was found to have a significant relationship with injury index ( $R = -0.31$ ,  $P < 0.05$ ). When pooled within each group, some interesting patterns have emerged (Fig. 8). Two wild barleys (the most tolerant group) had highest Na<sup>+</sup> concentration in leaves under control condition but lowest Na<sup>+</sup> concentration response to moderate salinity ( $P < 0.05$ , Fig. 8 A-B). These two wild varieties also had the highest K<sup>+</sup> content in their leaf sap when grown under saline conditions (Fig. 8E;  $P < 0.05$ ). Taken together, these results indicate that wild barleys have a better ability for Na<sup>+</sup> exclusion and K<sup>+</sup> retention in shoot. The tolerant group had substantially less Na<sup>+</sup> in leaves

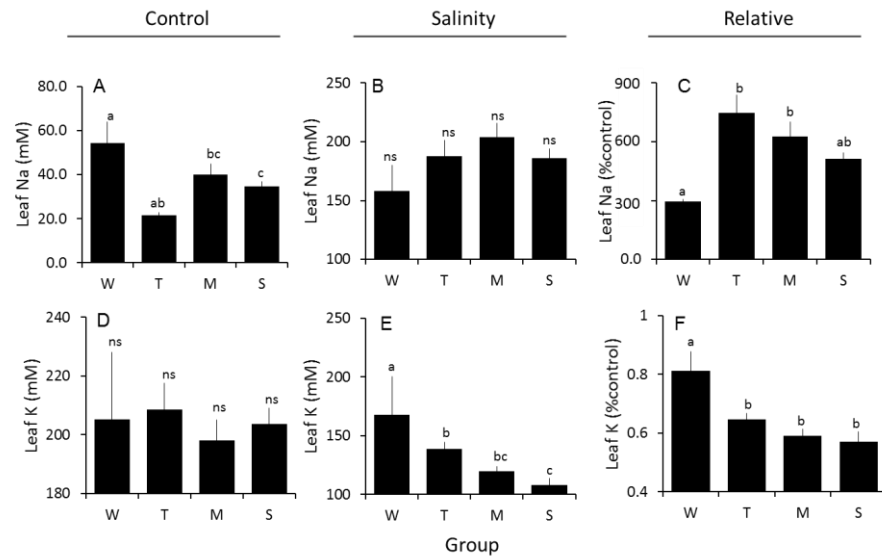
under control conditions ( $P < 0.05$ ; Fig. 8A) but showed the highest relative increase in leaf  $\text{Na}^+$  under saline conditions (Fig. 8C). For both absolute (Fig. 8E) and relative (Fig. 8F) values, leaf sap  $\text{K}^+$  concentration declined in a sequence  $W > T > M > S$ .



**Fig. 7.** Shoot  $\text{Na}^+$  content (mM) of 46 barley varieties grown under control and salt stress (200 mM NaCl for 35 days) conditions. Mean  $\pm$  SE ( $n = 6$ ).



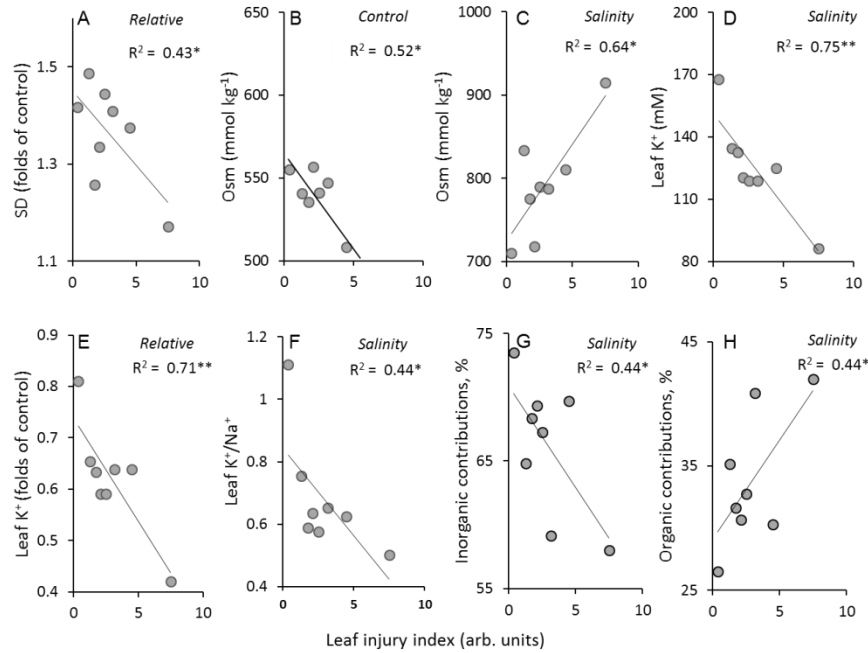
**Fig. 8.** Shoot K<sup>+</sup> content (mM) of 50 barley varieties grown under control and salt stress (200 mM NaCl for 35 days) conditions. Mean  $\pm$  SE (n = 6).



**Fig. 9.** Mean shoot ionic content (A-C, sodium; D-E, potassium) for plants clustered according to their salinity stress tolerance. Cluster 1 = wild barley (W; scoring index < 1); Cluster 2 = tolerant (T; scoring index 1 – 2); Cluster 3 = moderately tolerant (M; scoring index 2 – 3); Cluster 4 = sensitive (S; scoring index > 3). Data is mean  $\pm$  SE (n = 6). A, D – control; B, E – salinity; C, F – relative (% control).

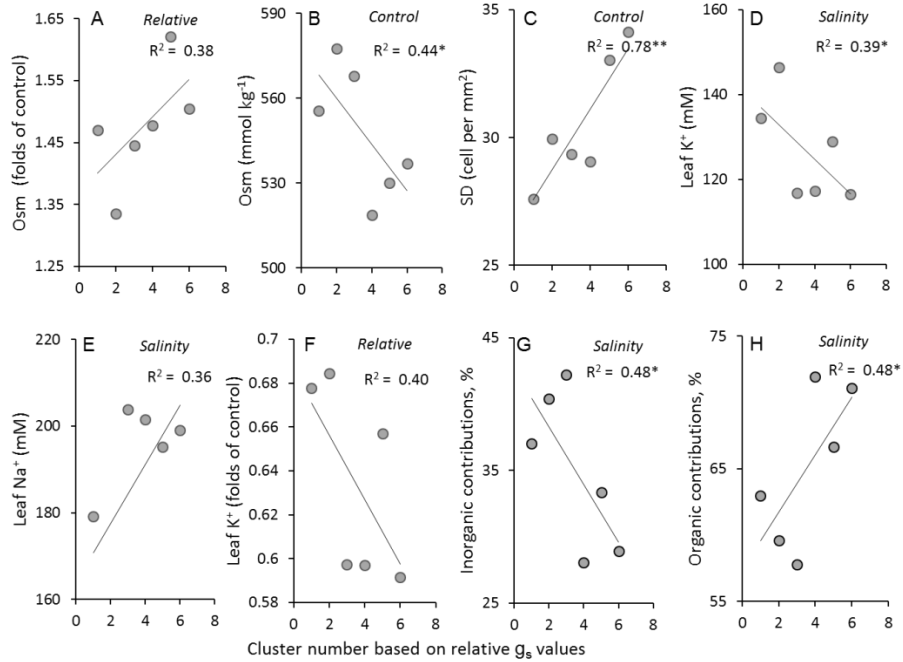
Despite some obvious trends, most of the data reported in Figs 5 & 9 were not significantly different, suggesting a big biological variability (most likely resulting from polygenic nature of salinity trait) within the same cluster. To overcome this problem, we have undertaken a finer clustering by dividing plants into 8 groups using the injury index. All physiological traits were then correlated with the injury index. Only those physiological traits which have significant correlations with leaf injury index are shown in Figure 10. In brief, a significant ( $P < 0.05$ ) relationship was found between the extent of leaf injury and changes in stomata density (biggest increase in tolerant varieties; Fig. 10A), osmolality (higher Osm values in control; Fig. 10B) but lower Osm values under saline conditions (Fig. 10C) in tolerant varieties), leaf sap potassium content under saline conditions (both absolute (Fig. 10D) and relative (Fig. 10E); higher in tolerant varieties), leaf  $K^+/Na^+$  (Fig. 10F; higher in tolerant varieties). Tolerant varieties also had significantly higher contribution of inorganic osmolytes (Fig.

10G) and lower contribution of organic osmolytes (Fig. 10H) towards their osmotic adjustment under saline conditions. Taken together, these results suggest that tolerant barley varieties might be able to maintain more efficient photosynthesis due to higher stomata density (Fig. 10A), as well as prominent  $K^+$  retention in leaves (Fig. 10D and Fig. 10E). Moreover, the ability of using surplus ion as ‘cheap’ osmolytes to maintain turgor pressure is the other distinction between tolerant and sensitive groups (Fig. 10G and Fig. 10F). Energetic-consuming synthesis of organic osmolytes brings growth retardation, resulting in plant hypersensitive to salinity.



**Fig. 10.** Correlation between physiological characteristics and leaf injury index under saline conditions. All plants were divided into several clusters, and pooled injury score index was calculated for each cluster. Cluster #1  $\leq 0.5$ ; #2 = 1.0 – 1.5; #3 = 1.75 – 2.25; #4 = 2.25; #5 = 2.5 – 2.75; #6 = 3.0 – 3.5; #7 = 4.0 – 5.0; #8 = 5.75 – 9.5; SD – stomata density, OSM – osmolality. Only those physiological traits which have significant correlations with leaf injury index are shown. Asterisks indicate a level of significance for a two-tailed test (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ).

We have also addressed a possible link between stress-induced changes in stomatal conductance and other physiological indices measured. For doing that, all 46 barley genotypes were clustered into 6 major groups according to their relative  $g_s$  values (% control) under saline conditions. Cluster #1 included most sensitive varieties ( $g_s$  values between 30 and 40% of control), while cluster #6 included varieties in which  $g_s$  was barely affected by salinity (80 to 100% of control). Only those physiological traits which have significant correlations with relative  $g_s$  values are shown in Figure 11. Relative leaf sap osmolality (Fig. 11A), stomata density in control (Fig. 11C), sap  $\text{Na}^+$  concentration under saline conditions (Fig. 11E), and contribution of inorganic osmolytes towards osmotic adjustment (Fig. 11H) were all correlated positively with  $g_s$  sensitivity to salinity stress (higher values in less susceptible cultivars). Four traits were correlated negatively with changes in  $g_s$ . These include leaf sap  $\text{K}^+$  concentration in saline plants (both absolute – Fig. 11D, and relative – Fig. 11F), leaf sap osmolality in control (Fig. 11B) and contribution of organic osmolytes towards osmotic adjustment under saline conditions (Fig. 11H).



**Fig. 11.** Correlation between physiological characteristics and relative  $g_s$  values (%control) of different groups. All plants were divided into several clusters based on relative  $g_s$  values (%control): Cluster #1 = 30 – 40% of control; #2 = 40 – 50%; #3 = 50 – 60%; #4 = 60 – 70%; #5 = 70 – 80%; #6 = 80 – 100%. Each point represents a pooled value for all genotypes within one cluster. Only those physiological traits which have significant correlations with relative  $g_s$  values are shown. Asterisks indicate a level of significance for a two-tailed test (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ).

## Discussion

### *Salinity stress tolerance in barley does not correlate with $g_s$ under moderate stress conditions*

In this work,  $g_s$  measurements were conducted on the youngest fully-expanded leaf which might explain values being slightly lower than previously reported in the literature. Another reason may be the fact that measurements were conducted during the winter season and thus the values measured may be related to plant's seasonal activity as well. Still, values between 50 and 100 mmol m<sup>-2</sup> s<sup>-1</sup> (as



reported in Fig. 3) have been previously reported for barley grown under both control and saline conditions (e.g. Jiang *et al.* 2006; Adem *et al.* 2014).

According to our results shown in Fig. 5 (A – C) and Fig. 10, no correlation was found between  $g_s$  and plant salinity injury index. Previous research showed that the main factor of growth under moderate salinity is the stomatal limitation of photosynthesis (Brugnoli and Lauteri 1991; Munns and Tester 2008; Rajendran *et al.* 2009). By screening 14 barley genotypes, Jiang *et al.* (2006) found a positive correlation between  $g_s$  values under saline conditions and shoot dry matter. However, these authors used only 33 mM NaCl to treat their plants; this is below the accepted salinity threshold of 4 dS/m (or 40 mM NaCl). Similarly, James *et al.* (2008) observed a positive relationship between  $g_s$  and relative growth rate of 50 durum wheat genotypes cultivated in hydroponic system with 150 mM NaCl (James *et al.* 2008). However, the salinity stress lasted only for 10 days only, and this is a period during which osmotic component of the salt stress is said to dominate (Munns 2002). Thus, these results make it difficult to make an unequivocal conclusion on a relationship between  $g_s$  and salinity stress tolerance. Our results from the large-scale barley varieties treated with 200 mM NaCl for five weeks showed no correlation between  $g_s$  and salinity tolerance. One potential explanation is that reduced stomatal conductance would not necessarily cause the reduction in rates of photosynthesis per unit leaf area in salt-treated plants (James *et al.* 2002), because the smaller but thicker salt-affected leaves (compared to control plants) have higher chloroplast density per unit leaf area. Hence, the unchanged photosynthesis promises the sufficient carbon assimilation, which could be a strategy adopted by plants to resist salinity. Also, the overall salinity stress tolerance in this work was estimated as the leaf injury index in plants treated with 320 mM NaCl for about 3-4 weeks. To deal with such high levels of salt plant may require higher tissue tolerance which may not necessarily correlate with osmotic stress tolerance (a dominant component at mild salinities; Munns *et al.* 2010). Thus, the lack of direct correlation between changes in  $g_s$  and leaf injury index (Fig. 10) is hardly surprising.

Another interesting observation in this study was a positive correlation between stomata density under saline conditions and plant salt tolerance (Fig. 10A). These observations are consistent with previous reports on a halophyte species of quinoa, in which SD correlated positively with biomass in 14 quinoa genotypes treated with 400 mM NaCl for 8 weeks (Shabala *et al.* 2012). However, in the latter study, salinity stress has resulted in a decrease in the stomata density in quinoa; this was also confirmed by independent study from another laboratory (Orsini *et al.* 2011). In our work, however, a salinity-induced increase in stomata density (Fig. 4) was found. This difference may be attributed to two factors. First, the level of salt stress differed by two-fold (400 vs 200 mM). Second, halophytes have highly efficient mechanisms for vacuolar Na<sup>+</sup> sequestration and display a pronounced succulency when grown under highly saline conditions; this effect is less pronounced in glycophytes. Thus, the observed difference may be explained by the fact that quinoa pavement cells increased their size thus pushing stomata further apart (hence, reducing SD) while barley cells were less efficient in doing this.

Interestingly, a clear trend was found in stomata density in four barley groups under control conditions ( $W < T < M = S$ ; Fig. 5D). These observations offer two clues. First, it appears that under control conditions salt-tolerant genotypes have naturally lower stomata density. This may be important for plants to improve their water-use efficiency (WUE). Second, increasing stomata in salt-tolerant barley genotypes under salinity is an optimal strategy for barley to maximum CO<sub>2</sub> assimilation. Wild barley genotypes generally have better survival performance when exposed to extreme saline conditions compared to cultivated cultivars; however, their yield performance is barely satisfactory, even under control conditions. Stomata, as the mediator of gas exchange, play a critical role in CO<sub>2</sub> assimilation and carbon fixation in photosynthesis, which finally contribute to plant biomass and yield. Hence, stomata density might be one of the constraints causing yield penalty in wild barley genotypes. The increase in stomata density observed in salt-treated barley leaves may be due to the change in cell anatomy: smaller leaf area, resulting in a higher number of stomata per unit leaf area. The possible explanation for positive correlation

between SD (%control) and plant salt tolerance is that tolerant plants can regulate ion flux into/out of guard cells more efficiently. However, more evidence is required to clarify the underlying mechanism of this phenomenon, e.g. the signal pathway mediating the increase of stomata density and which signal molecule or protein or protein complex is involved in the pathway.

***Salinity tolerance in barley correlates with the predominant use of inorganic osmolytes for osmotic adjustment***

Plant adaption to salinity requires mechanisms dealing with osmotic adjustment and avoiding ion toxicity. The relative importance of these components depends strongly on the severity of stress. If the stress is mild, the ion toxicity can be avoided by efficient Na<sup>+</sup> exclusion or sequestration (Quintero *et al.* 2008; Mian *et al.* 2011), and the osmotic component may dominate. To maintain leaf expansion growth shoot cells need to maintain positive turgor under hyperosmotic conditions in the rhizosphere. This requires osmotic adjustment that can be achieved by either increase in production of so-called compatible solutes, or by increased uptake of inorganic ions. It is still argued, however, which option is preferable for plant to confer salinity stress tolerance (reviewed in Hasegawa *et al.* 2000; Serraj and Sinclair 2002; Shabala and Shabala 2011). Consistent with previous results from our laboratory on few selected varieties (Chen *et al.* 2007), a negative correlation between contribution of organic osmolytes and salinity tolerance/stomatal conductance (Fig. 10H & 11H) was found. Moreover, a positive association between the contribution of inorganic osmolytes and  $g_s$  under saline condition (%control; Fig. 11F) confirmed that salinity tolerance correlates with the predominant use of inorganic osmolytes for osmotic adjustment. It is estimated that the energy cost of organic osmolytes production is at least 10 times higher compared with inorganic ion uptake, with between 30 and 109 molecules of ATP required for the autotrophic biosynthesis of one molecule of a different compatible solute, while one molecule of ATP should suffice for the accumulation of two K<sup>+</sup> and two Cl<sup>-</sup> ions (Oren 1999). Given the wide range of genotypes used in this study (46 in total), it is strongly suggestive that salinity tolerance in barley is associated with the genotype's

ability to avoid energy-consuming synthesis of organic osmolytes and avoid yield penalties by relying on the use of inorganic ions (especially  $\text{Na}^+$ ) as an energetically efficient strategy.

Also, as shown in Fig 11C, SD under control conditions correlates positively with relative changes in  $g_s$  under saline stress. This may be explained by the fact that these (osmo-tolerant) varieties have higher transpiration rate and, therefore, will be capable to deliver more  $\text{Na}^+$  to the shoot to be used for osmotic adjustment purposes. In a separate study, a significant positive ( $R^2 = 0.45$ ;  $P < 0.05$ ) correlation was found between an increase in shoot sap osmolality and sap  $\text{Na}^+$  content (Min Zhu, unpublished data). Because of this, substantial amounts of  $\text{Na}^+$  can be utilized as the energetically cheap osmoticum to maintain proper turgor pressure in plant cells, leaving more  $\text{K}^+$  in leaves available for essential metabolic processes (a negative correlation between  $\text{K}^+$  content and  $g_s$ ; Fig. 11D & 11F).

***Salinity tolerance correlates with  $\text{K}^+$  retention but not  $\text{Na}^+$  exclusion from the shoot***

Potassium is one of the most abundant and important inorganic cation in cytosol in plants, acting as a dominant counterion to equilibrate the negatively charged proteins and nucleic acids as well as being involved in enzyme activation, protein synthesis stabilization, membrane potential formation, maintenance of turgor pressure and cytosolic pH homeostasis, and mediating all types of plant movements (Shabala 2003; V ́ery and Sentenac 2003; Dreyer and Uozumi 2011; Ansch ́utz *et al.* 2014; Ch ́riel *et al.* 2014). However, elevated  $\text{Na}^+$  content in the cytosol can be toxic due to the physiochemical similarities between  $\text{Na}^+$  and  $\text{K}^+$  leading to a competition for transport and catalytic sites that normally bind the essential  $\text{K}^+$  (Tester and Davenport 2003; Chen *et al.* 2008; Cuin *et al.* 2008). The application of  $\text{K}^+$  fertilizers can ameliorate detrimental effects of salinity on plant performance when challenged by salinity stress (Cakmak 2005; Shabala and Pottosin 2014).

In this study, the fact that more tolerant plants constrained significantly higher  $K^+$  levels in their leaves under saline conditions (Fig. 9F; Fig. 10D &E) may indicate that these plants may naturally have higher capacity for  $K^+$  uptake and thus accumulate more  $K^+$  under non-saline conditions (Fig. 9D, the tolerant group has higher leaf  $K^+$  concentration). Furthermore, even without grouping, relative leaf  $K^+$  under moderate salinity stress has a significant negative correlation with salinity tolerance ( $R = -0.31$ ,  $P < 0.05$ ; Table 2). It is known that the concentration of cytosolic  $K^+$  is approximately the same in all species (Leigh and Tomos 1993; Dreyer and Uozumi 2011); thus the size of the vacuolar  $K^+$  pool might be the most likely reason. A salinity-induced reduction in leaf  $K^+$  content in all the barley genotypes could be observed when exposed to salinity (Fig. 8), which is detrimental to normal plant cell metabolism. Hence, the larger the vacuolar  $K^+$  pool is, the lower the leaf injury index will be. This result is consistent with the previous study in our laboratory (Wu *et al.* 2014).

### **Wild barley: a ‘stand-alone’ group**

Wild barley (*H. spontaneum*) has evolutionally adapted to a broad range of environments and formed rich genetic diversities for salt tolerance (Wu *et al.* 2013), generating the potential to use wild barley as a source for salt- and drought-resistant alleles for mechanism research and breeding purposes (Garthwaite *et al.* 2005; Nevo and Chen 2010). The aforementioned fact that barley tolerant genotypes have lower stomata density but the values of  $g_s$  among the four groups is not significantly different under control (Fig. 5) indicates they may have bigger stomata aperture under none-saline conditions. Under abiotic stress conditions such as salinity or drought, abscisic acid (ABA) can induce stomatal closure (Freundl *et al.* 2000; Hose *et al.* 2000). ABA-induced stomatal closing depends on cytosolic alkalization (Irving *et al.* 1992); this effect can occur in isolated membrane patches and appears to function by increasing the number of  $K^+_{out}$  channels available for activation (Miedema and Assmann 1996). Furthermore, increased external pH decreases  $K^+_{in}$  channel activity (Hedrich *et al.* 1995) and increases activation of a guard cell localized  $K^+_{out}$  channel (Ache *et al.* 2000). Two of the wild barley genotypes in this paper were found to have

the highest leaf  $K^+$  content under saline conditions (Fig. 9E & 9F), suggesting that the ability of deactivating  $K^+_{out}$  channels and activating  $K^+_{in}$  channels in wild barley might be better than in the cultivated barley genotypes, because higher leaf  $K^+$  content prevents the cytosol from being alkalized and inhibiting ABA-induced stomatal closure.

Another interesting phenomenon is that these two wild barleys were found to have the highest leaf  $Na^+$  content under non-saline conditions but lowest increase in  $Na^+$  content in leaf after exposure to salinity stress. These findings are suggestive that wild barley is highly efficient in controlling xylem  $Na^+$  loading to achieve optimal osmotic adjustment in the shoot by mean of  $Na^+$ . Recently we showed (Bose *et al.* 2014) that salt-sensitive pea plants display a gradual pattern of xylem  $Na^+$  loading while more tolerant barley species rapidly upload  $Na^+$  into the xylem within several hours of salinity exposure and then maintain xylem  $Na^+$  concentration at more or less constant level. It appears that wild barley is even more efficient in doing this. A two-fold higher leaf sap  $Na^+$  content under control conditions (Fig. 9A) indicates that wild barley rely heavily on  $Na^+$  transport to the shoot to maintain cell turgor. When exposed to saline conditions, the overall increase of shoot  $Na^+$  is only 65% of that in cultivated varieties (Fig. 9A & B) while  $g_s$  values (and, hence, transpiration rate) are not significantly different. This indicates a highly efficient control of xylem  $Na^+$  loading in wild barley. From this point of view, wild barley can be used as the ideal model for research on the mechanism of salinity tolerance; the similarities between halophytes and wild barley (less stomata but bigger aperture on leaf surface; better ability of  $K^+$  retention; Shabala *et al.* 2012) will offer insights into mechanisms behind salinity tolerance.

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## **Chapter 3: Evaluating predictive values of various physiological indices for salinity stress tolerance in wheat<sup>2</sup>**

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### **Abstract**

Soil salinity is a worldwide issue that affects agricultural production. The understanding of mechanisms by which plants tolerate salt stress is crucial for breeding varieties for salt tolerance. In this work, a large number of wheat (*Triticum aestivum* and *Triticum turgidum*) cultivars were screened by using a broad range of physiological indices. A regression analysis was then used to evaluate the relative contribution of each of these traits towards the overall salinity tolerance. In general, most of the bread wheats showed better Na<sup>+</sup> exclusion that was associated with higher relative yield. Leaf K<sup>+</sup>/Na<sup>+</sup> ratio and leaf and xylem K<sup>+</sup> contents were the major factors determining salinity stress tolerance in wheat. Other important traits included high xylem K<sup>+</sup> content, high stomatal conductance, and low osmolality. Bread wheat and durum wheat showed different tolerance mechanisms, with leaf K<sup>+</sup>/Na<sup>+</sup> content in durum wheat making no significant contributions to salt tolerance, while the important traits were leaf and xylem K<sup>+</sup> contents. These results indicate that Na<sup>+</sup> sequestration ability is much stronger in durum compared with bread wheat, most likely as a compensation for its lesser efficiency to exclude Na<sup>+</sup> from transport to the shoot. We also concluded that plant survival scores under high

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salt stress can be used in bread wheat as a preliminary selection for Na<sup>+</sup> exclusion gene(s).

**Key words:** wheat; salt tolerance; sodium; potassium; stomatal conductance; stomata density

### Introduction

Soil salinisation is a global issue that affects plant growth and limits agricultural production. Approximately 20% of the world's cultivated land, which accounts for over 6% of the world total area, is threatened by salinity (FAO 2008). In Australia, two-thirds of agricultural land area has a potential for transient salinity (Munns and James 2003). The most efficient way to minimise the detrimental effects of salinity on crop production is the development of the varieties with high salinity tolerance.

Wheat is one of the most important cereal crops worldwide in term of production and utilization. It provides approximately one fifth of the total calorific consumption of the world's population (FAO 2010). Among wheat species, durum wheat is generally less tolerant to salt stress than bread wheat (Munns *et al.* 2006; Munns and Tester 2008), mainly because of high rates of Na<sup>+</sup> accumulation and poor K<sup>+</sup>/Na<sup>+</sup> discrimination (Asch *et al.* 2000; Chen *et al.* 2007a; Genc *et al.* 2007). In addition to Na<sup>+</sup> exclusion, a large number of other mechanisms contribute to salt tolerance (Brugnoli and Lauteri 1991; Rivelli *et al.* 2002; Chen *et al.* 2007b); each of these may be controlled by a large number of genes. Accordingly, most researchers agree that the best way to proceed with breeding would be via pyramiding different useful physiological traits. However, in spite of substantial efforts, the outcomes are still disappointingly poor; due to the physiological and genetic complexity of this trait, the lack of reliable screening tools and, most importantly, the lack of a comprehensive understanding of the mechanisms behind salinity tolerance.



Most of the previous studies on salt tolerance mechanisms are based on a rather limited number of varieties (Brugnoli and Lauteri 1991; Chen *et al.* 2007b). The results can be affected by both plant's tolerance to salt stress and genetic background of the selected varieties. The small sample number is clearly not good enough to convince plant breeders that certain traits can be used as key selecting criteria for salt tolerance. Moreover, very often the choice of the screening method was driven by the convenience (both in terms of time and costing) of the method rather than the actual physiological importance of a particular trait. The ones preferred by breeders include relative water content, germination rate, coleoptile length, stem and radicle length, dry and wet weight of roots and shoots (Mano and Takeda 1997; Chen *et al.* 2005; Tajbakhsh *et al.* 2006; Chen *et al.* 2008; Siahsar *et al.* 2009), leaf injury and reduction of CO<sub>2</sub> assimilation (James *et al.* 2002; Munns *et al.* 2010), loss of chlorophyll and damage to the photosynthetic apparatus (Krishnaraj *et al.* 1993), Na<sup>+</sup> exclusion from the shoot (Garcia *et al.* 1995), K<sup>+</sup>/Na<sup>+</sup> discrimination (Asch *et al.* 2000) and Cl<sup>-</sup> exclusion (Rogers and Noble 1992) and K<sup>+</sup> flux under salinity stress (Chen *et al.* 2005). Leaf injury and plant survival rate under relatively high salinity stress (Sayed 1985; Zhou *et al.* 2012; Xu *et al.* 2013) has also been used, and preliminary results on barley showed a positive correlation between plant survival rates under high salinity stress (320 mM NaCl) and grain yield under relatively lower salinity stress (200 mM NaCl) (Zhu *et al.* 2015). However, screening varieties for survival under extreme salinity stress could bring the risk of selecting against productivity, as a salt tolerant genotype screened under a high-salinity environment alone might not be productive (Richards 1983; Munns *et al.* 2006; El-Hendawy *et al.* 2009). Even so, neither comparison nor correlation between survival score index and crop yield has been studied using sufficient number of genotypes to meet statistical criterion. Long-term experiments are expected to be more reliable for screening plants for their salinity tolerance since the early response to salinity stress is driven mainly by the osmotic effect while salt-specific effect requires time to develop (Munns 1993; Munns *et al.* 1995; Munns and James 2003; Smethurst and Shabala 2003). So, the question remains: which physiological traits make the biggest

contribution towards the overall salinity tolerance (measured as relative yield under salt stress), and which parameters should be measured and which can be omitted as having low predictive value? This question was addressed in this work, by using a large number of durum and bread wheat accessions.

### Materials and Methods

#### *Plant materials*

Forty nine wheat cultivars (*Triticum aestivum* and *Triticum turgidum*), comprising 25 bread and 24 durum wheats, were obtained from the Australian Winter Cereal Collection and used in this study. The full list of cultivars is given in Table 1.

#### *Yield trial*

Yield trials were conducted under the glasshouse conditions in Launceston (Tasmania) in 2012. The day/night temperatures were about  $24/16 \pm 2^\circ\text{C}$ . Six seeds of each genotype were sown in a 4 L pot filled with a standard potting mixture (see Chen *et al.* 2007c for details). Seven days after sowing, a salinity treatment was started by watering plants with a 150 mM NaCl solution. The treatment was applied several times until the solution running out of the pot reached a salt level of 150 mM and  $\text{Na}^+$  levels in the soil were maintained at constant 150 mM level. The control pots were irrigated with domestic tap water and grown in the same glasshouse with three replications (Fig. 1). Salinity treatment lasted until plants were harvested for grain yield.

**Table 1** The list of wheat varieties used in this study

	<i>Bread wheat</i>	<i>Durum wheat</i>
<b>1</b>	Berkut	Wollaroi
<b>2</b>	Cranbrook	Caparoi
<b>3</b>	Drysdale	Hyperno
<b>4</b>	Excalibur	Jandaroi
<b>5</b>	Gladius	Kalka
<b>6</b>	Halberd	Tamaori
<b>7</b>	Krichauff	13953
<b>8</b>	Kukri	Alex
<b>9</b>	Opata	Aus 12746
<b>10</b>	Baart 46	Aus 16469
<b>11</b>	Sokol	Aus 19762
<b>12</b>	340	Biskri ac2
<b>13</b>	Belgrade 3	C 250
<b>14</b>	H7747	Citr 7792
<b>15</b>	India 38	Citr 7805
<b>16</b>	Iran 118	Covelle
<b>17</b>	Iraq 43	Jori
<b>18</b>	Iraq 50	Odin
<b>19</b>	Kharchia 65	Purple grain
<b>20</b>	Persia 118	Tehuacan 60
<b>21</b>	Persia 21	Timilia
<b>22</b>	Persia 6	Towner
<b>23</b>	Titmouse S	Zulu
<b>24</b>	Westonia	Seville 20
<b>25</b>	Janz	



**Fig. 1.** Yield trial in a glasshouse conducted in 2012.

### *Assessment of physiological traits*

The experiment was conducted in 2012 (March to June) in a glasshouse with day/night temperatures of approximately  $24/16 \pm 2^{\circ}\text{C}$ . Thirty seeds of each genotype were sown in 40-L poly (vinyl) chloride (PVC) containers filled with potting mixture. After germination, the trial was treated with a 150 mM NaCl solution (see above for details). Five weeks after the treatment, treated and control plants were assessed for various physiological traits described below.

#### *Stomatal conductance*

The first fully-expanded leaf was measured using a Decagon Leaf Porometer (Decagon Devices, Inc.). Measurements were conducted from seven plants of each cultivar exposed to salinity and controls. The highest and the lowest values were removed during analyses and the remaining data was averaged.

### *Stomatal density:*

The abaxial leaf surface was coated with clear nail varnish. The dried layer of the nail varnish was then peeled off using tweezers and placed on a glass slide. The number of stomata was counted for each field of view. For each of genotype/treatment, 3 imprints from 3 biological replicates were analysed.

### *Leaf sap osmolality and Na<sup>+</sup> and K<sup>+</sup> content*

The youngest fully-expanded leaf was harvested and quickly frozen in an Eppendorf tube. To measure ion content and osmolality, the leaf was defrosted and the sap extracted by hand-squeezing the leaf samples as described elsewhere (Cuin *et al.* 2009). The sap was centrifuged at 8,000 rpm for 10 minutes to remove debris. An amount of ~20 µl of the collected supernatant was used to measure sap osmolality using a vapour pressure osmometer (Vapro; WescorInc, Logan, UT, USA). An additional 50 µl of the collected supernatant was mixed with 5 ml of distilled water and used for determination of K<sup>+</sup> and Na<sup>+</sup> concentration (in mM) using a flame photometer (Corning 410C, Essex, UK). Five replicates for each cultivar for both salt treated and control plants were assessed.

### *Xylem Na<sup>+</sup> and K<sup>+</sup> content*

Xylem sap samples were collected by cutting plant stems near the base (5-10 mm above the potting media) and inserting them (upside down) in a Scholander-type pressure chamber (Plant Moisture Systems, Santa Barbara, CA, USA). The cut end protruded by about 5-8 mm through the air-tight rubber compression gland. Pressure was applied by filling the chamber with compressed air. The resultant excreted xylem sap was immediately collected with a micropipette and stored in an Eppendorf tube. The collected samples were weighed (to an

accuracy of 0.1 mg, accurate to 4 digits after the decimal point using 0.1 mg-readability balance), diluted with double distilled water (typically, by 20-fold), frozen and kept at -18°C until Na<sup>+</sup> and K<sup>+</sup> contents were measured using a flame photometer (Corning 410C, Essex, UK).

#### *Plant survival*

Six seeds of each cultivar were sown in a 4 L pot filled with the standard potting mixture. The salt treatment used was similar to the one described above, with an exception that the salt concentration was increased to 320 mM. Plant survival was assessed by combining scores for leaf chlorosis and plant survival when most of the most sensitive cultivars were dead (0 = no symptoms of seedling damage; 10 = completely dead plants) (Suppl. Fig. 2). Controls were omitted in this case since it has been proven in our earlier experiments that different varieties grown in the absence of salt showed no symptoms of leaf chlorosis or dead leaves (Zhou *et al.* 2012).

#### *Statistical analysis*

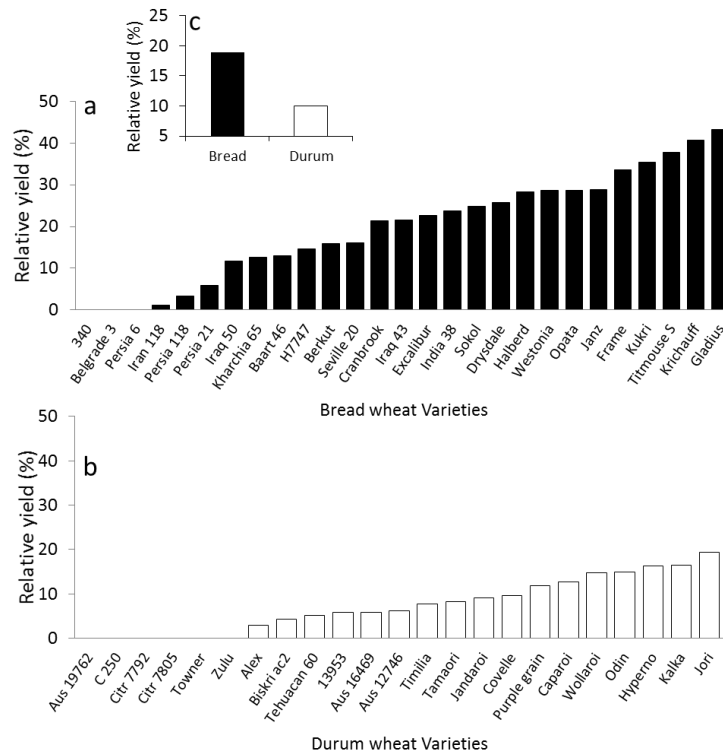
SPSS statistical 21 was used to find traits contributing significantly to relative yield. Four different scores of all the physiological traits were used for regression analysis. These include: values under control conditions (C), values under saline treatment (S), relative values under saline treatment (% control) (R), and a difference between salt treated plants and the respective controls (D).

### **Results**

#### *Yield performance of all 49 varieties under salinity stress*

The relative yields (% of control) of 49 wheat varieties under salt stress are shown in Fig 2. Yields of both bread and durum wheat decreased dramatically under 150 mM NaCl treatment. Bread wheat showed generally better tolerance than durum wheat with the average relative yield being  $19.8 \pm 2.60$  % for bread

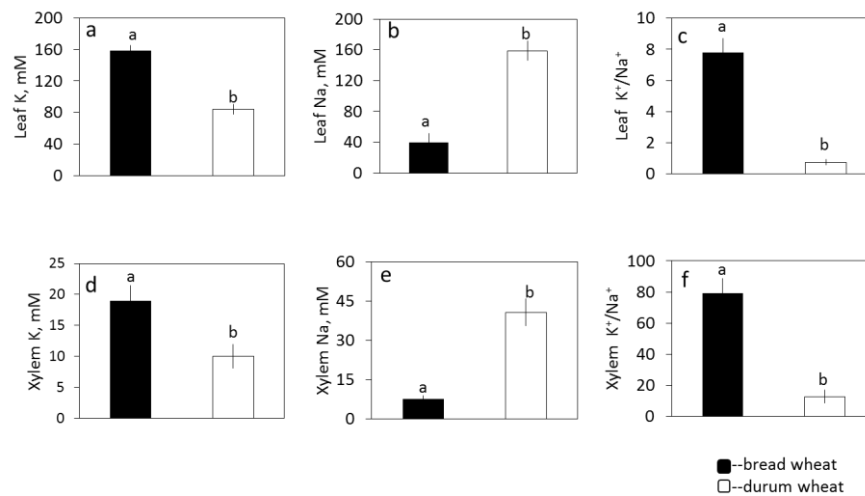
wheat compared with only  $8.1 \pm 1.26$  % for durum wheat. Varieties also showed significant differences in salt tolerance within each groups with the relative yield varying from 0 to 43.2% for bread wheat and from 0 to 19.3% for durum wheat (Fig. 2).



**Fig. 2.** Relative yield of 49 wheat varieties under 150 mM NaCl stress, a) 25 bread wheat genotypes; b) 24 durum wheat genotypes; c) comparison of relative yield in bread and durum wheat genotypes.

### *Correlation between relative yields and different physiological traits under salinity stress*

Most of the physiological traits assessed showed a wide range of variation among the varieties. Similar to the yield performance, the average performance of different physiological traits also varied between durum and bread wheat, with bread wheat showing significantly lower leaf and xylem  $\text{Na}^+$  content and higher leaf and xylem  $\text{K}^+$  content (Fig. 3).



**Fig. 3.** Differences of selected physiological traits between bread wheat and durum wheat grown under 150 mM NaCl for 5 weeks: a) leaf Na<sup>+</sup> concentration (mM); b) leaf K<sup>+</sup> concentration (mM); c) ratio of K<sup>+</sup>/Na<sup>+</sup> in leaf; d) xylem Na<sup>+</sup> concentration (mM); e) xylem K<sup>+</sup> concentration (mM); f) ratio of K<sup>+</sup>/Na<sup>+</sup> in xylem.

Table 2 shows correlations between various physiological traits and the relative yield of all 49 wheat genotypes. Stomatal conductance, stomatal density, leaf and xylem K<sup>+</sup> and Na<sup>+</sup> contents all showed significant (at  $P < 0.05$ ) correlations with the yield. Among the traits assessed, the leaf K<sup>+</sup>/Na<sup>+</sup> ratio was found to have the highest predictive value for the overall salt tolerance of all the varieties tested, showing the highest correlation with the relative yield ( $R^2 = 0.59$ ). For further analysis, varieties were grouped into durum and bread wheat clusters and analysed separately. For the bread wheat, leaf K<sup>+</sup>/Na<sup>+</sup> under salt stress was still the strongest determinant of salinity tolerance. Comparing the results from all 49 varieties tested, leaf and xylem K<sup>+</sup> content were less important in the tolerance of the bread wheat. In contrast, the tolerance of durum wheat heavily relied on leaf and xylem K<sup>+</sup> content while xylem Na<sup>+</sup> content showed no significant ( $P > 0.05$ ) correlation with the relative yields.



**Table 2** The correlations between relative grain yields under 150mM NaCl stress and different physiological traits

	<i>25 bread wheats</i>	<i>24 durum wheats</i>	<i>All 49 wheats</i>
Stomatal conductance- C	0.38*	0.49*	0.40**
Stomatal conductance - S	0.37	0.36	0.44**
Stomatal conductance - R	0.11	0.18	0.22
Stomatal conductance - D	-0.11	-0.25	-0.08
stomatal density - C	0.57**	0.17	0.52**
stomatal density -S	0.55**	0.44	0.56**
stomatal density - R	-0.04	0.52*	0.20
stomatal density - D	0.15	0.54**	0.33*
<i>osmolality - C</i>	-0.02	0.03	0.14
<i>osmolality - S</i>	-0.13	0.22	0.09
<i>osmolality - R</i>	-0.11	0.12	-0.07
<i>osmolality - D</i>	-0.13	0.18	-0.04
leaf K - C	0.28	0.19	0.30*
leaf K - S	0.44*	0.53**	0.64**
leaf K - R	0.30	0.52*	0.58**
leaf K - D	0.30	0.45*	0.57**
leaf Na - C	-0.12	0.29	-0.26
leaf Na - S	-0.54**	-0.43*	-0.65**
leaf Na - R	-0.48*	-0.29	-0.59**
leaf Na - D	-0.54**	-0.43*	-0.65**
xylem K - C	-0.16	-0.07	-0.16
xylem K - S	0.10	0.58**	0.41**
xylem K - R	0.20	0.44*	0.46**
xylem K - D	0.19	0.45*	0.45**
xylem Na - C	-0.26	0.21	-0.21
xylem Na - S	-0.25	-0.07	-0.41**
xylem Na - R	-0.06	-0.29	-0.28
xylem Na - D	-0.23	-0.11	-0.40**
leaf K/Na - S	0.74**	0.53**	0.77**
xylem K/Na - S	0.22	0.46*	0.51**

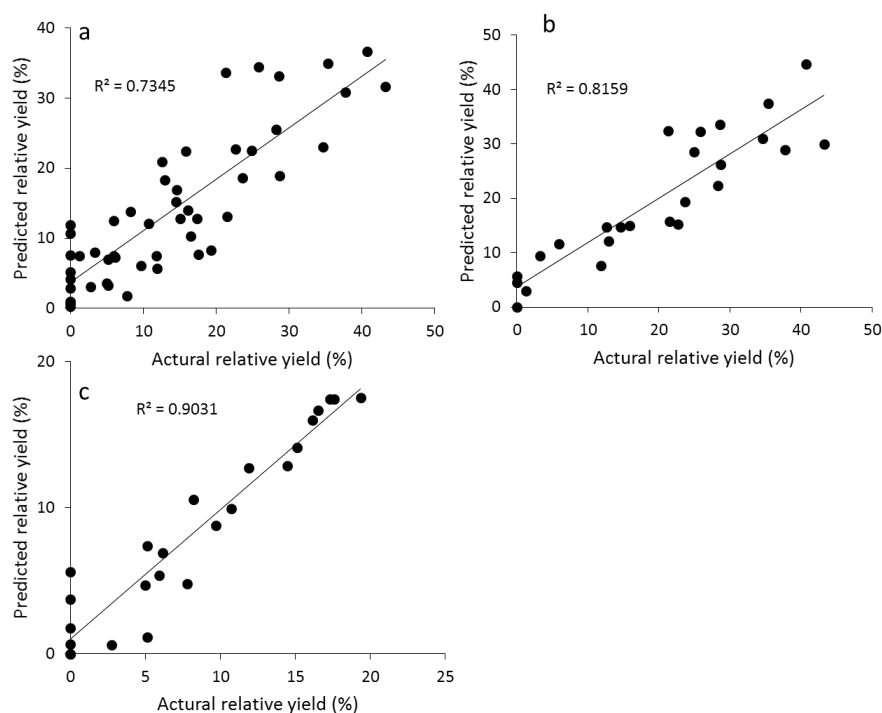
$r_{(0.05, 48)} = 0.28$ ;  $-r_{(0.05, 48)} = 0.36$ ;  $r_{(0.05, 21)} = 0.42$ ;  $r_{(0.05, 21)} = 0.53$ ;  $r_{(0.05, 25)} = 0.38$ -  
 $r_{(0.05, 25)} = 0.49$

### *Physiological traits that showed significant contribution to salt tolerance*

Many physiological traits were found to correlate to each other and showed significant correlation with the relative yield (Table 3). To find the most important trait/s that made major contributions to salt tolerance, a linear regression analysis was conducted on all 49 wheat varieties, as well as being applied separately to bread wheat and durum wheat clusters. All the values for different traits were standardised before the analysis. As shown in Table 3, when the analysis was based on the full set of 49 varieties, only leaf  $K^+$  (D), leaf  $K^+/Na^+$  (S),  $g_s$  (S),  $g_s$  (D) and stomatal density (R) showed significant contributions to the yield, with leaf  $K^+/Na^+$  (S) being the most important trait. Together, these five traits determined more than 73% of the relative yield (Fig. 4a). Further analysis was conducted for each of the bread and durum wheat clusters. For bread wheat, eight physiological traits showed significant contributions to the tolerance and determined 82% of yield variation (Fig. 4b). Leaf  $Na^+$  and  $K^+$  contents were the major contributors to the yield, followed by  $g_s$ , stomatal density, and xylem  $Na^+$  (Table 3). In contrast, leaf and xylem  $K^+$  contents were major contributors to tolerance in durum wheat (Table 3). Even though osmolality had no significant correlation with salt tolerance in durum wheat, it made a significant contribution to the tolerance; most likely due to the high correlation between osmolality and stomatal conductance. Very high correlations were found in durum wheat between actual yield and the yields predicted using different physiological traits ( $R^2 = 0.90$ , Fig. 4c).

**Table 3** Coefficients of physiological traits which showed significant contribution to relative grain yield under salt stress

	<i>25 bread wheats</i>	<i>24 durum wheats</i>	<i>All 49 wheats</i>
Intercept	46.5	85.1	-22.6
Leaf Na – C	34.3		
leaf K – D		15.8	3.8
leaf K – R	-42.0		
Xylem K - S	-13.0	29.3	
Xylem K - C	22.0		
xylem K - R		-44.1	
xylem K - D		-23.2	
xylem Na – R	7.0		
leaf K/Na - S	-27.8		10.2
Stomatal conductance - C	14.1		
Stomatal conductance - D		5.6	2.4
Stomatal conductance - S		3.7	4.8
Stomatal density -R	-21.2	18.2	15.5
OSM –C		-38.6	
OSM –R		-43.7	



**Fig. 4.** Correlations between actual relative yield and the yield predicted from those physiological traits that made significant contributions to salinity tolerance (Table 2). a) All 49 varieties; b) bread wheat; c) durum wheat. (The predicted yield values were obtained from the equation using the coefficients of physiological traits which showed significant contribution to relative yield under salt stress, Table 3).

In additional statistical analysis, both bread and durum wheat were each further divided into four groups: sensitive (S – relative yield ranging from 0 – 5% for durum wheat and 0 – 10% for bread wheat); moderately sensitive (MS – relative yield ranging 5 to 10% for durum wheat and 10 to 20% for bread wheat); moderately tolerant (MT - relative yield ranging 10 to 15% for durum wheat and 20 to 30% for bread wheat) and tolerant (T - relative yield ranging from >15% for durum wheat and >30% for bread wheat) (Table 4 and 5). The average values of selected physiological traits which showed significant contributions to relative grain yield in the above regression analysis are shown in Fig. 5 and 6. Again, leaf  $K^+/Na^+$  was more important in bread wheat than that in durum wheat. Xylem  $K^+/Na^+$  ratio was also important for salt tolerance in durum wheat. For

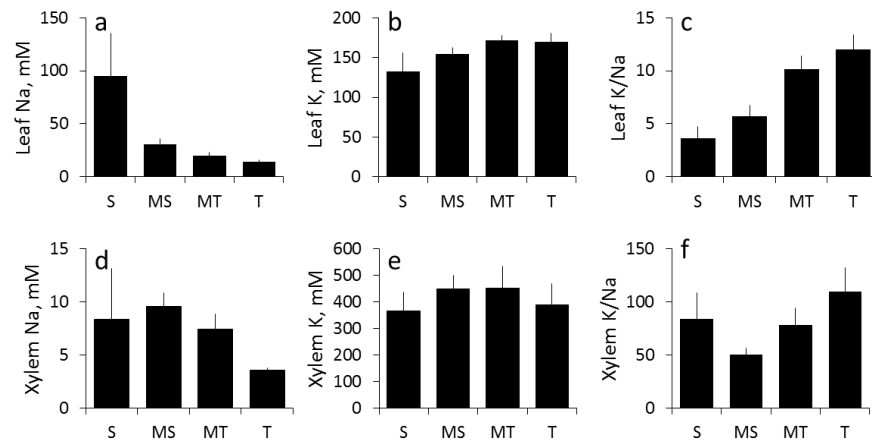
bread wheat, leaf Na<sup>+</sup> content was one of the key determinants for salt tolerance while in durum wheat, the difference in Na<sup>+</sup> content between salt-sensitive and salt-tolerant genotypes was much less.

**Table 4** Genotypes ranking according to salinity tolerance in 25 bread wheat genotypes

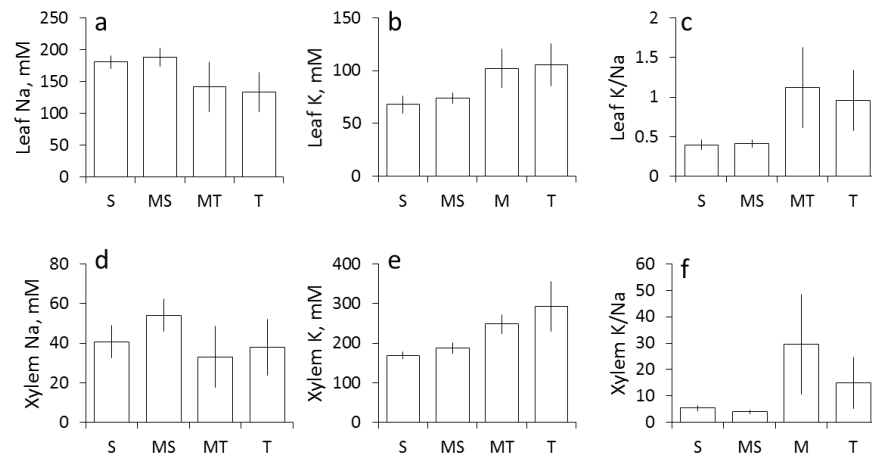
	<i>Varieties</i>	<i>Relative yield (%)</i>		<i>Varieties</i>	<i>Relative yield (%)</i>
<b>S</b>	340	0.00	<b>MT</b>	Cranbrook	21.29
	Belgrade 3	0.00		Iraq 43	21.49
	Persia 6	0.00		Excalibur	22.68
	Iran 118	1.25		India 38	23.67
	Persia 118	3.29		Sokol	24.91
	Persia 21	5.90		Drysdale	25.79
<b>MS</b>	Iraq 50	11.80	<b>T</b>	Halberd	28.28
	Kharchia 65	12.58		Westonia	28.63
	Baart 46	12.94		Opata	28.72
	H7747	14.55		Janz	28.87
	Berkut	15.83		Kukri	35.39
				Titmouse S	37.75
				Krichauff	34.71
				Gladius	43.25

**Table 5** Genotypes ranking according to salinity tolerance in 24 durum wheat genotypes

	<i>Varieties</i>	<i>Relative yield (%)</i>	<i>Varieties</i>	<i>Relative yield (%)</i>
<b>S</b>	Aus 19762	0.00	Tehuacan 60	5.12
	C 250	0.00	13953	5.90
	Citr 7792	0.00	Aus 16469	5.93
	Citr 7805	0.00	Aus 12746	6.14
	Towner	0.00	Timilia	7.79
	Zulu	0.00	Tamaori	8.19
	Alex	2.93	Jandaroi	9.05
	Biskri ac2	4.39	Covelle	9.70
<b>MT</b>	Purple grain	11.89	Seville 20	16.14
	Caparoi	12.64	Hyperno	16.32
	Wollaroi	14.85	Kalka	16.49
	Odin	14.97	Jori	19.33



**Fig. 5.** Mean shoot ionic content for 25 bread wheat genotypes clustered according to their salinity stress tolerance. Cluster 1 = Sensitive (S; relative yield < 10); Cluster 2 = moderately sensitive (MS; relative yield 10 – 20); Cluster 3 = moderately tolerant (MT; relative yield 20 – 30); Cluster 4 = tolerant (T; relative yield > 30). Data is mean  $\pm$  SE (n = 6).



**Fig. 6.** Mean shoot ionic content for 24 durum wheat genotypes clustered according to their salinity stress tolerance. Cluster 1 = Sensitive (S; relative yield < 5); Cluster 2 = moderately sensitive (MS; relative yield 5 – 10); Cluster 3 = moderately tolerant (MT; relative yield 10 – 15); Cluster 4 = tolerant (T; relative yield > 15). Data is mean  $\pm$  SE (n = 6).

### *Plant survival under high salt pressure (320 mM NaCl)*

In a separate set of experiments, higher salt stress (320 mM NaCl) was applied to all varieties, to test their survival rate (Fig. 7). Two weeks after salt stress, the lower leaves of susceptible varieties started to wilt, which is a sign of osmotic stress resulting from salinity stress. After that, leaf chlorosis and necrosis gradually developed, and some plants started to die as the stress continued. When most plants of the susceptible varieties were dead (approximately 5 weeks after commencing the treatment), the damage index was scored. On average, bread wheat showed significantly better tolerance than durum wheat (Fig. 8a), with the seedling damage scores ranging from 1.5 to 6.0 for bread wheat and from 3.5 to 7.5 for durum wheat. Damage scores of plant seedlings measured upon 320 mM NaCl treatment showed significant correlation with the relative yield under 150 mM NaCl treatment ( $R^2 = 0.12$ ,  $P < 0.05$ ). Also, much stronger correlation was found between seedling damage scores and leaf  $\text{Na}^+$  and  $\text{K}^+$  content. As shown in Fig. 8b and 8c, all 49 varieties can be split into two distinct groups. Nearly all of the varieties in the low  $\text{Na}^+$  content group and high  $\text{K}^+$  content group were bread wheats, while nearly all durum wheat fell into the other group.

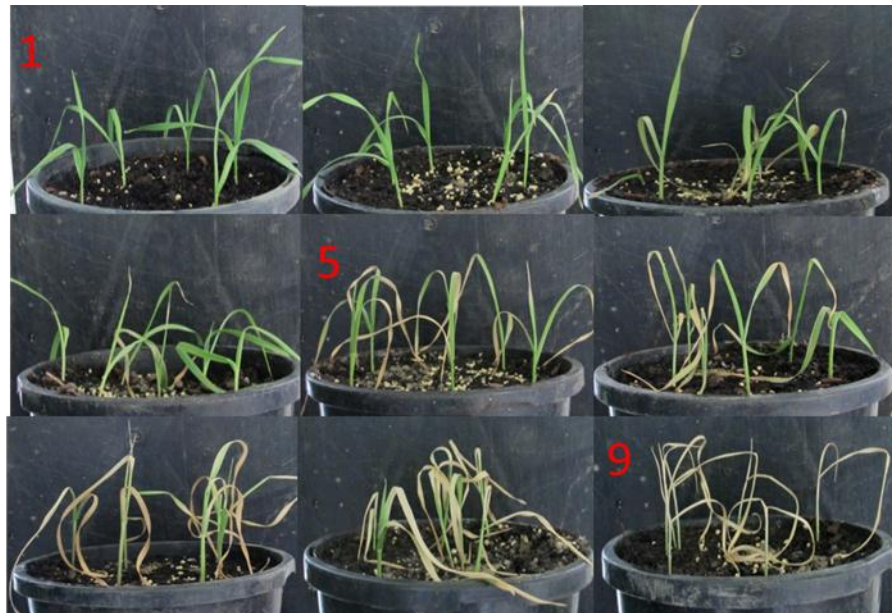
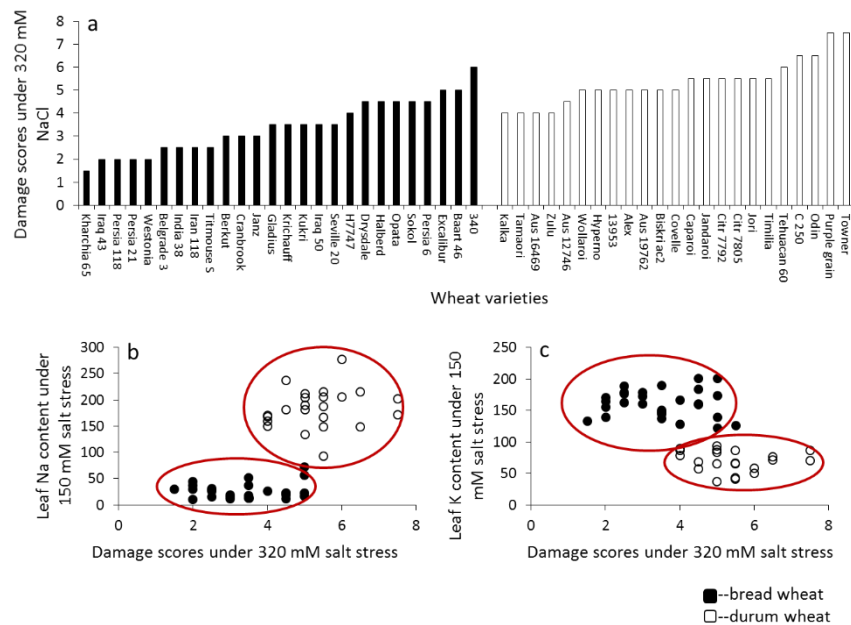


Fig. 7. Plant seedling damage scores under high salinity stress.





**Fig. 8.** Plant survival under higher concentration (320 mM) and its relationship with leaf K<sup>+</sup> and Na<sup>+</sup> contents.

### Discussions

#### *Leaf K<sup>+</sup>/Na<sup>+</sup> ratio has the highest prediction value for salt tolerance in wheat*

Elevated Na<sup>+</sup> concentration in the cytosol can cause a degradation of chlorophyll and inhibit the normal functioning of a large number of enzymes and proteins, resulting from the competition by Na<sup>+</sup> for K<sup>+</sup> binding sites (Clarkson and Hanson 1980; Shabala 2003; V éry and Sentenac 2003; Shabala and Cuin 2008; Dreyer and Uozumi 2011; Ansch ütz *et al.* 2014; Ch érel *et al.* 2014). It is not surprising, therefore, that a plant's ability to maintain an optimal K<sup>+</sup>/Na<sup>+</sup> ratio has long been cited as a key feature of salinity tolerance (Chhipa and Lal 1995; Asch *et al.* 2000; Munns 2002; Tester and Davenport 2003; Cuin *et al.* 2011). In our experiment with 49 wheat varieties (25 bread and 24 durum), leaf K<sup>+</sup>/Na<sup>+</sup> ratio showed the highest correlation with salt tolerance, determining nearly 60% of the phenotypic variation. However, the optimal tissue K<sup>+</sup>/Na<sup>+</sup> ratio can be achieved by both restricting Na<sup>+</sup> accumulation in the shoot, or improved K<sup>+</sup>

retention in leaf mesophyll. While a reduced rate of  $\text{Na}^+$  loading into the xylem was traditionally considered as one of the most crucial features conferring salinity tolerance in glycophytes (Munns and Tester 2008; Tester and Davenport 2003; Shabala *et al.* 2010; Shabala *et al.* 2013), our data revealed no significant correlation between xylem  $\text{Na}^+$  content and salinity tolerance, when analysed separately between durum and bread wheats (Table 2). Both xylem and leaf  $\text{K}^+$  correlated highly positive with the overall salinity stress tolerance in each of the clusters (Table 2). Hence, it can be concluded that a plant's ability to deliver  $\text{K}^+$  to the shoot and retain it in the mesophyll, rather than excluding  $\text{Na}^+$  from the shoot, makes a major contribution towards maintaining an optimal leaf  $\text{K}^+/\text{Na}^+$  ratio and affecting the overall salinity tolerance in wheat. Consistent with this notion are our previous results on barley showing that tolerant genotypes were more efficient in maintaining higher xylem  $\text{K}^+/\text{Na}^+$  ratios under saline conditions (Shabala *et al.* 2010).

#### ***Higher stomatal conductance and density correlates with salt tolerance in wheat***

Another factor limiting plant growth under intermediate salinity is stomatal limitation of photosynthesis (Seemann and Critchley 1985; Everard *et al.* 1994). Osmotic stress imposed by salinity leads to reduced stomatal conductance (Munns and James 2003); this constraint dominates for a period of several weeks, until specific ion toxicity in leaves starts to play a major role. Under short-term, mild NaCl treatment, a reduction of stomatal conductance was found early in the life of the leaf, positive correlations were found between  $g_s$  and shoot dry matter in barley (Jiang *et al.* 2006), cotton and beans (Brugnoli and Lauteri 1991). Significant correlations between salt tolerance and both stomatal conductance and stomatal density were also found in the current study (Table 2). Varieties with higher stomatal conductance and stomatal density tended to have better salt tolerance (Table 3), despite the fact that this strategy might come with the danger of delivering high quantities of  $\text{Na}^+$  to the shoot with the transpiration stream. It may be suggested, therefore, that in tolerant varieties this trait is complemented by higher tissue tolerance originating from either more efficient vacuolar  $\text{Na}^+$

sequestration, or better K<sup>+</sup> retention in leaf mesophyll, or both. In this context, both latter traits were found to be essential to confer salinity tolerance in a facultative halophyte species of *Chenopodium quinoa* (Bonales-Alatorre et al. 2013), and a strong positive correlation ( $R^2 = 0.41$ ,  $P < 0.001$ ) was found between NaCl-induced K<sup>+</sup> efflux from leaf mesophyll and an overall salinity tolerance in a large number of wheat accessions in our recent experiments (Wu et al. 2014).

#### ***Plant survival test as a convenient screening tool***

Plant survival scores recorded in this study are based on the plant growth under extreme saline conditions (320 mM in this study). While this concentration may be deemed un-physiologically high for wheat, it is relatively fast and simple and has been used in many studies (Zhou et al. 2012; Xu et al. 2012), and showed a significant correlation with grain yield of plants grown under moderate (150 mM NaCl) conditions ( $R^2 = 0.12$ ;  $P < 0.05$ ). The physiological rationale behind this approach is that, under such severe conditions, the genetic variability in Na<sup>+</sup> exclusion from uptake will play a lesser role, and tissue tolerance will become the dominating trait. The possible caveat here is that such screening for genotypic survival under extreme salinity might make selection against productivity and thus is risky (Richards 1983; Munns et al. 2006; Munns and Tester 2008; El-Hendawy et al. 2009). This issue should be kept in mind if the method is used in breeding programs. However, tissue tolerance is a major contributing mechanism, both in the naturally salt-tolerant species such as halophytes (Flowers and Colmer 2008; Shabala and Mackay 2011; Shabala 2013), and also in salt-tolerant glycophyte species such as barley (Adem et al. 2014). Thus, we believe this trait should be targeted more actively in other cereals, and specifically in wheat, unless varieties are grown under very mild saline conditions.

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**Chapter 4: Physiological and molecular mechanisms mediating xylem Na<sup>+</sup> loading in barley in the context of salinity stress tolerance<sup>3</sup>**

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**Abstract**

Time-dependent kinetics of xylem Na<sup>+</sup> loading was investigated using a large number of barley genotypes contrasting in their salinity tolerance. In short term, accumulation of Na<sup>+</sup> in the xylem sap of salt-tolerant genotypes was higher than in their salt-sensitive counterparts. Here, xylem Na<sup>+</sup> reached its peak 5 days after salt application and then declined. Salt-sensitive varieties were less efficient in controlling xylem Na<sup>+</sup> loading and showed a gradual increase in the xylem Na<sup>+</sup> content over the time. To understand underlying ionic and molecular mechanisms, net fluxes of Ca<sup>2+</sup>, K<sup>+</sup> and Na<sup>+</sup> were measured from the xylem parenchyma tissue in response to H<sub>2</sub>O<sub>2</sub> and ABA; both of them associated with salinity stress signalling. Our results indicate that NADPH oxidase-mediated apoplastic H<sub>2</sub>O<sub>2</sub> production acts upstream of the xylem Na<sup>+</sup> loading and is causally related to ROS-inducible Ca<sup>2+</sup> uptake systems in the root stelar tissue. It was also found that ABA regulates (directly or indirectly) the process of Na<sup>+</sup> retrieval from the xylem and the significant reduction of Na<sup>+</sup> and K<sup>+</sup> fluxes induced by bumetanide are indicative of a major role of chloride cation co-transporter (CCC) on xylem ion loading. Transcript levels of *HvHKT1;5\_like* and *HvSOS1\_like* genes in the root stele were observed to decrease after salt

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stress while there was an increase in *HvSKOR\_like* gene, indicating that these ion transporters are involved in primary Na<sup>+</sup>/K<sup>+</sup> movement into/out of xylem.

**Keywords:** xylem ion loading, sodium, H<sub>2</sub>O<sub>2</sub>, ABA, barley, salinity stress

### Introduction

Salinity tolerance is a complex multigenic trait, both genetically and physiologically (Flowers, 2004). It is estimated that, in transcriptional level, approximate 8% of all genes in plant are affected by salinity stress (Tester and Davenport 2003). Among the multiple physiological mechanisms conferring salinity tolerance, control of xylem ion loading and regulation of ionic exchange at the xylem-parenchyma boundary have often been named as central to salinity tolerance (Tester and Davenport 2003; Munns and Tester 2008). A hypothesis was suggested by Shabala (Shabala 2003; Shabala *et al.* 2013) that, in the initial stage of salinity stress, a certain amount of Na<sup>+</sup> will be quickly transported into shoot for the osmotic adjustment. After the latter is achieved, reducing Na<sup>+</sup> loading into xylem or retrieval Na<sup>+</sup> from xylem would be a ‘wise’ choice for plant to avoid Na<sup>+</sup> accumulation in shoot, particularly in leaf tissue, where the photosynthetic activity could be disturbed by excessive cytosolic Na<sup>+</sup>. Also essential for plants is delivery of sufficient amounts of K<sup>+</sup> to the shoot, to maintain normal cell metabolism in photosynthetically active mesophyll cells. With the application of molecular biology techniques, several candidate genes of ion transporter relevant to xylem ion loading were characterized, such as Na<sup>+</sup>/H<sup>+</sup> antiporter (SOS1; Shi *et al.* 2002; Shi *et al.* 2003; Feki *et al.* 2011; Feki *et al.* 2014), high-affinity Na<sup>+</sup>/K<sup>+</sup>-permeable transporter (HKT; reviewed by Horie *et al.* 2009), chloride cation co-transporter (CCC; Colmenero-Flores *et al.* 2007), and Shaker-like outward channel (SKOR; Gaymard *et al.* 1998). All these transporters were found to potentially mediate xylem K<sup>+</sup> and Na<sup>+</sup> loading.

AtSOS1 has been identified as Na<sup>+</sup>/H<sup>+</sup> antiporters localised in epidermal cells at the root tip where they actively extrude Na<sup>+</sup> from cytosol into rhizosphere

(Shi *et al.* 2002). *SOS1* expression signal was also strong in the parenchyma root cells at the xylem/symplast boundary (Shi *et al.* 2002), where it may mediate xylem Na<sup>+</sup> loading and delivery to the shoot. Overexpression of *SOS1* in transgenic *Arabidopsis* has been shown to improve salt tolerance (Shi *et al.* 2003). Feki *et al.* have identified the *TdSOS1* gene from durum wheat, and the heterologous expression of *TdSOS1* in a yeast strain lacking endogenous Na<sup>+</sup> efflux proteins showed complementation involving cation efflux (Feki *et al.* 2011). On the contrary, HKT transporters could retrieve Na<sup>+</sup> from the xylem and contribute to Na<sup>+</sup> exclusion from leaves when expressed in the xylem parenchyma cells (Munns and Tester 2008; Horie *et al.* 2009). HKTs were first cloned from wheat (*Triticum aestivum*) and characterised as a high-affinity K<sup>+</sup> uptake systems (Schachtman *et al.* 1992). Subsequently, the HKT family was found to show functional diversity. Sub-family 1 comprises transporters that are permeable to Na<sup>+</sup> only, and sub-family 2 comprises transporters that are permeable to both K<sup>+</sup> and Na<sup>+</sup> (Rodriguez-Navarro and Rubio 2006). In sub-family 2, *TaHKT2;1* has been found to mediate high-affinity Na<sup>+</sup>-K<sup>+</sup> co-transport and also preferred Na<sup>+</sup>-selective low-affinity Na<sup>+</sup> transport in *Xenopus laevis* oocytes and yeast exposed to 1 mM NaCl (Rubio *et al.* 1995; Gassmann *et al.* 1996). *AtHKT1;1* in the *Arabidopsis* root is considered to be involved in Na<sup>+</sup> unloading from the xylem before it reaches the shoot (Davenport *et al.* 2007). Similar evidence was accumulated for the same function for the member of the closely related *HKT1;5* gene family in rice (Ren *et al.* 2005) and wheat (Garthwaite *et al.* 2005; James *et al.* 2006; Byrt *et al.* 2007; James *et al.* 2011; Byrt *et al.* 2014).

In *Arabidopsis*, Shaker-like K<sup>+</sup> Outward Rectifying (SKOR) channel has been shown to be expressed in parenchyma cells facing xylem vessels (Véry *et al.* 2014). The *skor* mutant appear to be impaired in potassium delivery to shoot, indicating that the *SKOR* gene encodes the stelar K<sup>+</sup> outward rectifier. *SKOR* expression is strongly inhibited by ABA, supporting the hypothesis that control of K<sup>+</sup> translocation to the shoot is a part of the plant response to water stress (Gaymard *et al.* 1998). Another important player in xylem ion loading is Na<sup>+</sup>:K<sup>+</sup>:Cl<sup>-</sup> co-transporter (abbreviated as CCC; Colmenero-Flores *et al.* 2007),

which was found to have preferential expression in the root and shoot vasculature at the xylem/symplast boundary. With the observation of higher and lower Cl<sup>-</sup> amounts in shoots and roots respectively in *atccc* mutant under saline condition, it was suggested that AtCCC is involved in the long-distance Cl<sup>-</sup> transport. At the same time, CCC-mediated Cl<sup>-</sup> loading into the xylem may also bring Na<sup>+</sup> and K<sup>+</sup> ions into it. Yet, the role of CCC in xylem loading under salinity stress remains unknown. This issue was addressed in this study using pharmacological approach (measuring net K<sup>+</sup> and Na<sup>+</sup> fluxes followed application of bumetanide, a specific inhibitor of vertebrate Na<sup>+</sup>:K<sup>+</sup>:Cl<sup>-</sup> co-transporter (Starremans *et al.* 2003).

While the molecular identity of candidate transporters potentially mediating xylem Na<sup>+</sup> and K<sup>+</sup> loading has been revealed, the process of their regulation is much less understood. Although the inhibition by ABA of channel-mediated efflux of K<sup>+</sup> into xylem has been well documented (Cram and Pitman 1972; Roberts 1998; Gilliam 2002), little is known about ABA control of xylem Na<sup>+</sup> loading. One interesting observation, however, is that the addition of ABA stimulated H<sup>+</sup> extrusion into xylem, so could potentially enhance active xylem loading via stimulation of Na<sup>+</sup>/H<sup>+</sup> antiporter activity in the plasma membrane of xylem parenchyma cells (Clarkson and Hanson 1986; Hall *et al.* 2006). Besides ABA, H<sub>2</sub>O<sub>2</sub> could act as potential second messenger regulating xylem ion loading. However, information on this matter is also scarce. Meanwhile, ROS were shown to activate Na<sup>+</sup>-permeable non-selective cation channels (NSCCs) in both roots (Demidchik and Tester 2002; Demidchik *et al.* 2003; Carvalho *et al.* 2010; Zepeda-Jazo *et al.* 2011) and leaves (Pei *et al.* 2000; Kwak *et al.* 2003). Activation of the K<sup>+</sup>-permeable outward rectifying GORK channel by OH<sup>-</sup> was observed in protoplasts isolated from *Arabidopsis* roots (Demidchik *et al.* 2010). Revealing the possible role of ABA and H<sub>2</sub>O<sub>2</sub> on regulating xylem ion loading was another aim of this study.

The main objective of this work was to answer the following questions: is there a difference in the kinetics of xylem ion loading under salinity stress between salt-tolerant and salt-sensitive genotypes? How is this loading controlled at physiological level? What is the molecular identity of the transport systems

behind this process, and to what extent changes in their expression pattern contribute to this process? The overall goal of this work was to offer a comprehensive insight into mechanisms of ion loading/unloading in xylem in context of salinity stress.

### Materials and Methods

#### *Plant materials*

The seeds of 46 barley cultivars (*Hordeum vulgare* and *Hordeum spontaneum*) were obtained from the Australian Winter Cereal Collection and from the barley genotype collection of Zhejiang and Yangzhou Universities in China. Those cultivars show genotypic variation in salinity tolerance under saline conditions and was clustered into four groups (wild barley; tolerant; moderate tolerant; sensitive) according to their performance under extreme salt stress (please refer to Chapter 2/ Table 1 for the details of 46 barley varieties used).

#### *Growth conditions and treatment*

The experiment was conducted in 2011 (July to November) in a glasshouse in Mt Pleasant laboratories in TIA (Launceston) with day/night temperatures of approximately 24/16 ± 2 °C. Plants were grown in a 40-L poly (vinyl) chloride (PVC) containers (two genotypes per container) filled with the fertilised standard potting mix (see Chen *et al.*, 2007 for details). 30 seeds were planted for each genotype. One week after sowing the emerged seedlings were treated immediately with 200 mM NaCl for 5 weeks. For the kinetics of xylem Na<sup>+</sup> loading assay, 200 mM NaCl was immediately applied to emerged seedlings and the xylem sap was extracted as described below on 1 day, 2 days, 5 days, and 10 days after the treatment.



### ***Leaf sap Na<sup>+</sup> and K<sup>+</sup> content***

The youngest fully-expanded leaf was harvested, placed into an Eppendorf tube and quickly frozen. To measure ion content and osmolality, the leaf was thawed and the sap extracted by hand-squeezing the leaf samples as described elsewhere (Cuin *et al.* 2009). The sap was centrifuged at 8,000 rpm for 10 minutes to remove debris. An additional 50 µl of the collected supernatant was mixed with 5 ml of distilled water and used for determination of K<sup>+</sup> and Na<sup>+</sup> concentration (in mM) using a flame photometer (Corning 410C, Essex, UK). Five replicates for each cultivar for both salt treated and control plants were assessed.

### ***Xylem Na<sup>+</sup> and K<sup>+</sup> content***

Xylem sap samples were collected by cutting plant stems near the base (5-10 mm above the potting media) and inserting them (upside down) in a Scholander-type pressure chamber (Plant Moisture Systems, Santa Barbara, CA, USA). The cut end protruded by about 5-8 mm through the air-tight rubber compression gland. Pressure (Approximately 10 bars for control plants and 25 bars for salt-affected plants.) was applied by filling the chamber with compressed air. The resultant excreted xylem sap was immediately collected with a micropipette and stored in an Eppendorf tube. The collected samples were weighed (to an accuracy of 0.1 mg, accurate to 4 digits after the decimal point using 0.1 mg-readability balance), diluted with double distilled water (typically, by 20-fold), frozen and kept at -18°C until Na<sup>+</sup> and K<sup>+</sup> contents were measured using a flame photometer (Corning 410C, Essex, UK).

### ***MIFE non-invasive ion flux measurements***

Net ion fluxes were measured using non-invasive ion-selective vibrating microelectrodes (the MIFE technique; University of Tasmania, Hobart, Australia). The principles of MIFE ion flux measurements are described in full elsewhere (Shabala *et al.* 1997), and all the details of microelectrodes fabrication and calibration are available in our previous publications (Shabala

and Shabala 2002; Shabala *et al.* 2006). Liquid ionic exchangers used in this work were commercially available ionophore cocktails (60031 for K<sup>+</sup>; 71176 for Na<sup>+</sup>; 95297 for H<sup>+</sup>; 21048 for Ca<sup>2+</sup>; all from Sigma). Three ion-selective microelectrodes (depends on different assays), were used in the same experiment. Electrode tips were aligned and positioned 40-50 µm above the stele.

### ***MIFE experimental protocols***

Seeds were surface sterilised with 10% commercially available bleach (King White, Victoria, Australia) for 10 minutes, rinsed thoroughly with deionised water, and then grown hydroponically for 3 days in the dark in aerated Basic Salt Medium (BSM) containing 0.1 mM CaCl<sub>2</sub> and 0.5 mM KCl (pH = 5.6; non-buffered). Seedlings were then transferred into fresh BSM containing 100 mM NaCl for 24 hours if needed (depending on experimental protocol).

For pharmacological experiments, the root stele from 3-day-old seedling (either non-salt treated or salt treated with 100 mM NaCl for 24 h) was mechanically isolated as described earlier (Shabala *et al.* 2010). An apical stelar segment was cut (the first 5-7 mm of the stele) and then immobilised in a Perspex chamber in BSM (with/without 10 mM thiourea or 20 µM DPI, respectively). Once the steady-state fluxes were reached (30-50 min after the immobilisation), measurements commenced. Net fluxes of ions (K<sup>+</sup>, Ca<sup>2+</sup> and H<sup>+</sup>) were measured for 5 to 10 min from root stele. Then treatment (either 10 mM H<sub>2</sub>O<sub>2</sub> or 10 µM ABA) was administered, and net fluxes were measured for further 30 min.

A so-called “recovery protocol” (Cuin *et al.* 2011) was used to quantify the activity of Na<sup>+</sup> efflux system in stelar root tissues. 3-day-old wheat seedlings were treated with 100 mM NaCl for another 24 h in the darkness, leading to significant accumulation of Na<sup>+</sup> in root tissues. The root stele peeled from salt-treated seedlings (3-day-old seedling grown under 100 mM NaCl for 24 h) was then put into a Petri dish with BSM (containing 20 mM NaCl) and left to recover overnight. After adaptation, the root was quickly but thoroughly rinsed with 10 mM CaCl<sub>2</sub> solution, to remove apoplastic NaCl. The root was then transferred

into a clean chamber containing Na<sup>+</sup>-free BSM solution (with/without 0.1 mM amiloride or 0.1 mM bumetanide). Net ion fluxes were measured from the stelar tissue approximately for 5-10 min.

### ***RNA extraction and RT-qPCR experiments***

Three barley cultivars (CPI, CM72 and Naso Nijo) were grown in BSM for 3 d and then transferred into BSM containing 100 mM NaCl for 24 h. Total RNA was extracted from ~100 mg of root stelar and epidermal tissue using the RNeasy Plant Mini Kit (Qiagen). First-strand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen), which includes the genomic DNA removal step. Relative transcript levels of three genes (*HvHKT1;5\_like*, *HvSKOR\_like* and *HvSOS1\_like*) and reference gene (*HvGAPdH2*) were assayed by real-time qPCR analysis using Qiagen Rotor-gene real-time PCR system. RT-qPCR conditions were as follows: 95 °C for 2 min, 95 °C for 5 s and 60 °C for 10 s (50 cycles). Amplified products were detected using QuantiNova SYBR Green PCR Kit (Qiagen). Each data point was determined in triplicate in each sample and presented as mean  $\pm$  SE (n = 6). For the detailed primer information, please refer to Table 1.

### ***Statistical analysis***

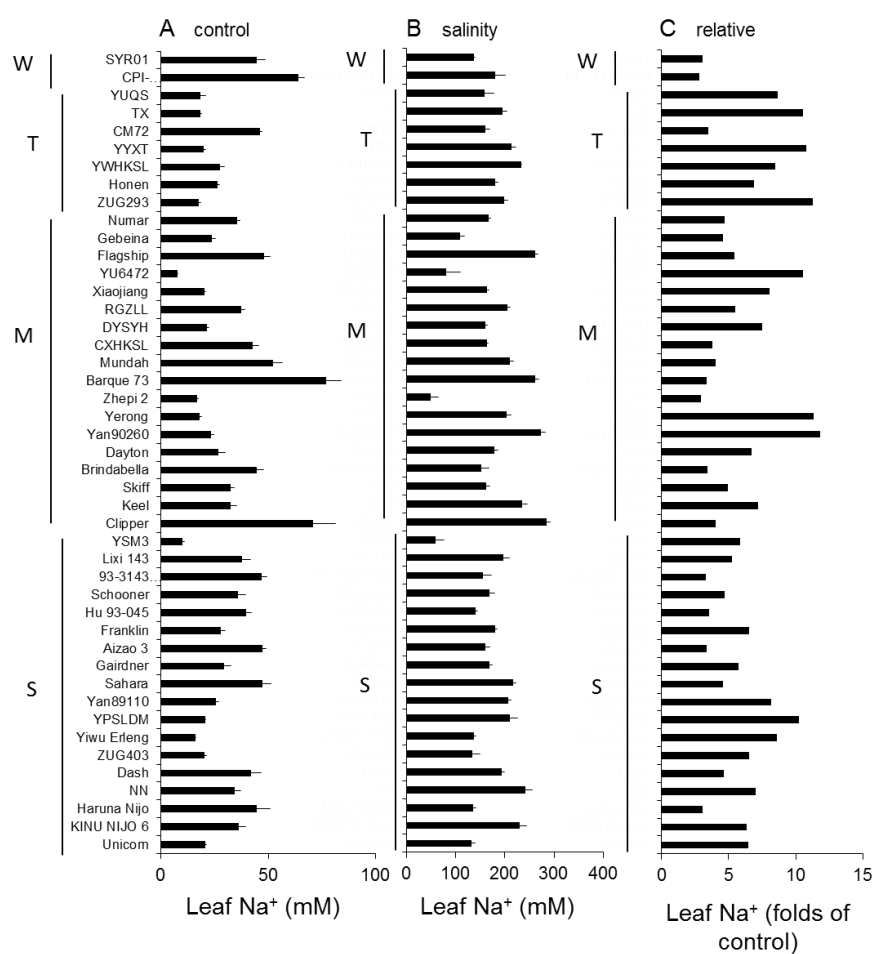
Data were analysed using IBM SPSS Statistics 21 for Windows. All results are given as means  $\pm$  SE. Then one-way ANOVA was used to calculate the significance of difference between the results. Difference low-case letter in each panel of the figures indicate significance at  $P < 0.05$  level.

Table 1 The Primers used in the gene expression study and their respective amplicton size.

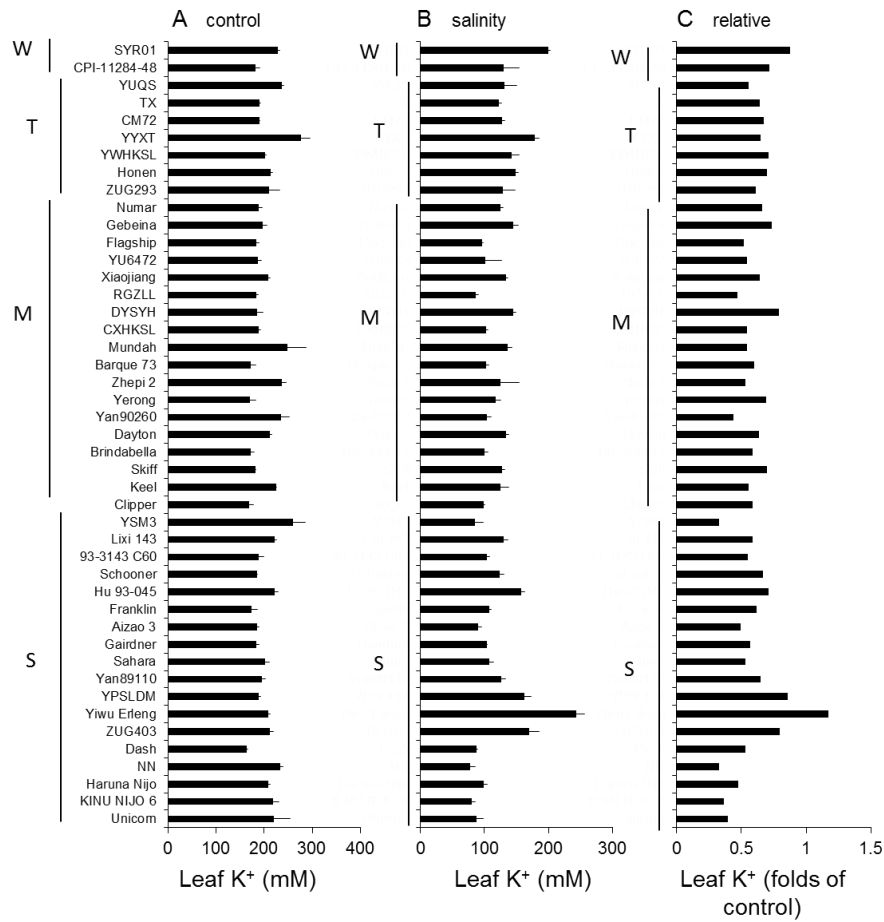
Gene Name	Forward Primer	Reverse Primer	Amplicton Size (bp)
<i>HvHKT1;5_like</i>	TCAAGGAGAC GGCGAAGGA	AGCACGAAGAG GACGAGGA	274
<i>HvSKOR_like</i>	GGCGTCAATA CTCTTCAGCAA	CAGCGTCTACC AGCAACTTC	215
<i>HvSOS1_like</i>	AAGCACAGCA AGTCGTATCC	GAGTTGTCATCT TCCGCTATCT	200
<i>HvGAPdH2</i>	GTGAGGCTGG TGCTGATTACG	TGGTGCAGCTA GCATTTGAGAC	Reference gene

### Results

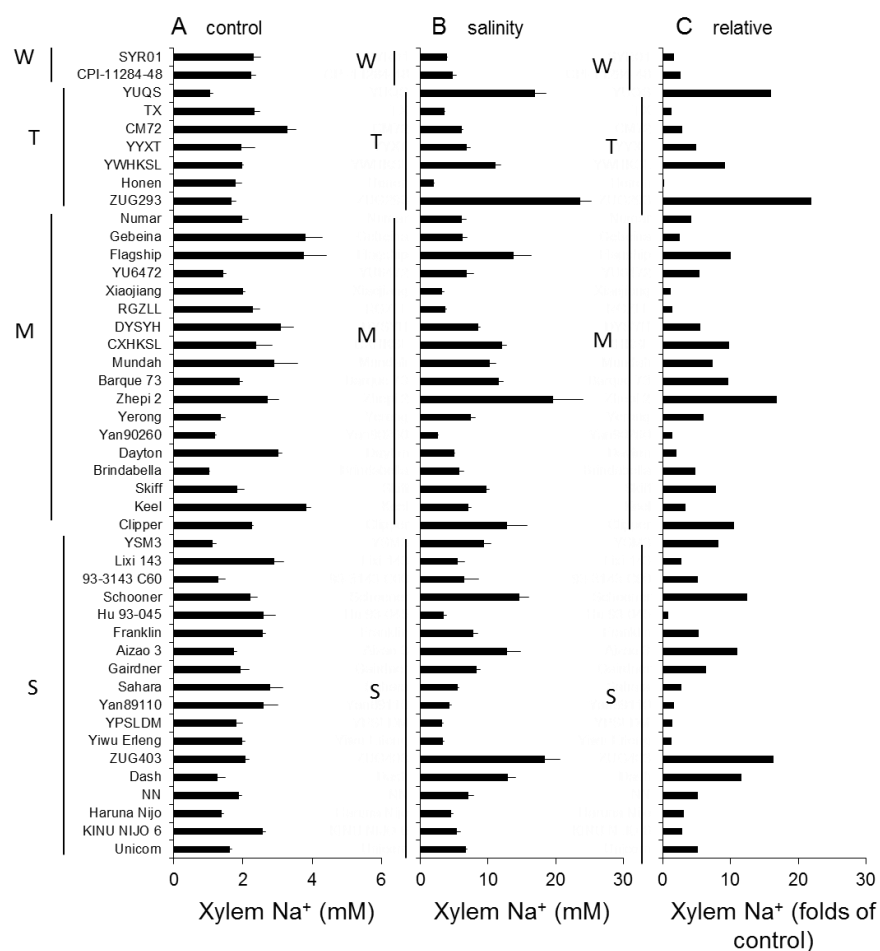
After 5 weeks of moderate salinity stress (200 mM NaCl), both leaf and xylem Na<sup>+</sup> concentration increased significantly ( $P < 0.05$ ) in all 46 barley genotypes, compared with control values (3 to 12 folds increase in the leaf, Fig. 1; and 0.8 to 22 folds increase in the xylem, Fig. 3). On the contrary, K<sup>+</sup> concentration in the leaf and the xylem decreased dramatically after plants were challenged by salinity (Fig. 2). Yet, there were some genotypes in which xylem K<sup>+</sup> concentration under saline condition was higher than control values (Fig. 4).



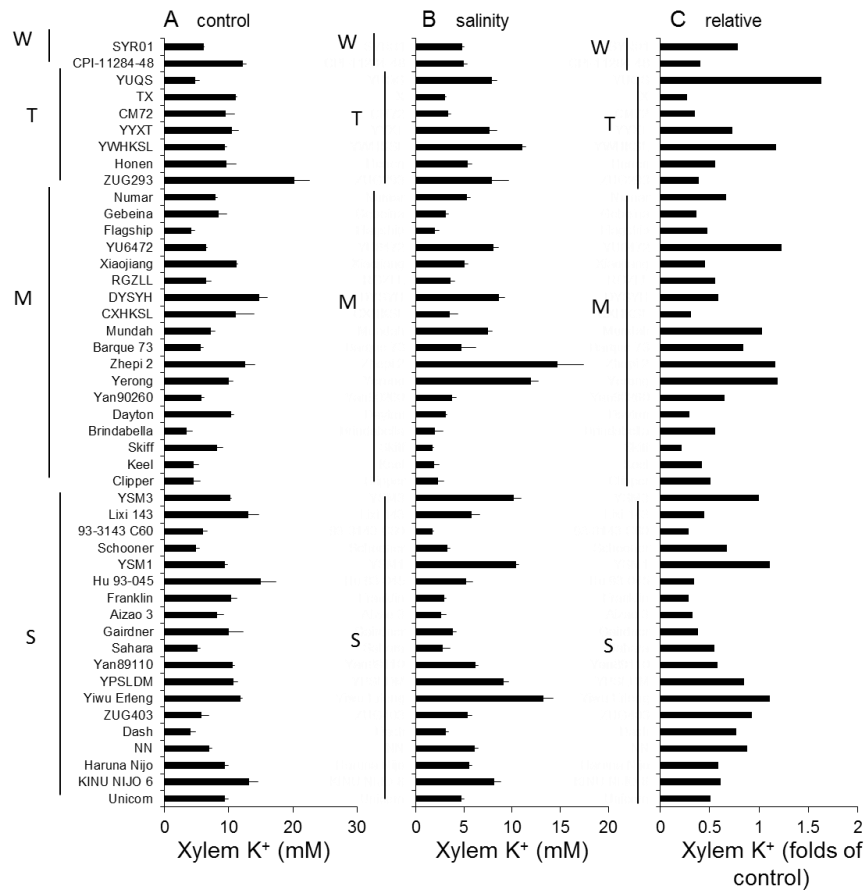
**Fig. 1.** Na<sup>+</sup> concentration in leaf (mM) of 46 barley genotypes grown under control and salt stress (200 mM NaCl for 5 weeks) conditions. Mean  $\pm$  SE (n = 6). A – Control; B – salinity; C – relative (folds of control).



**Fig. 2.** K<sup>+</sup> concentration in leaf (mM) of 46 barley genotypes grown under control and salt stress (200 mM NaCl for 5 weeks) conditions. Mean  $\pm$  SE (n = 6). A – Control; B – salinity; C – relative (folds of control).



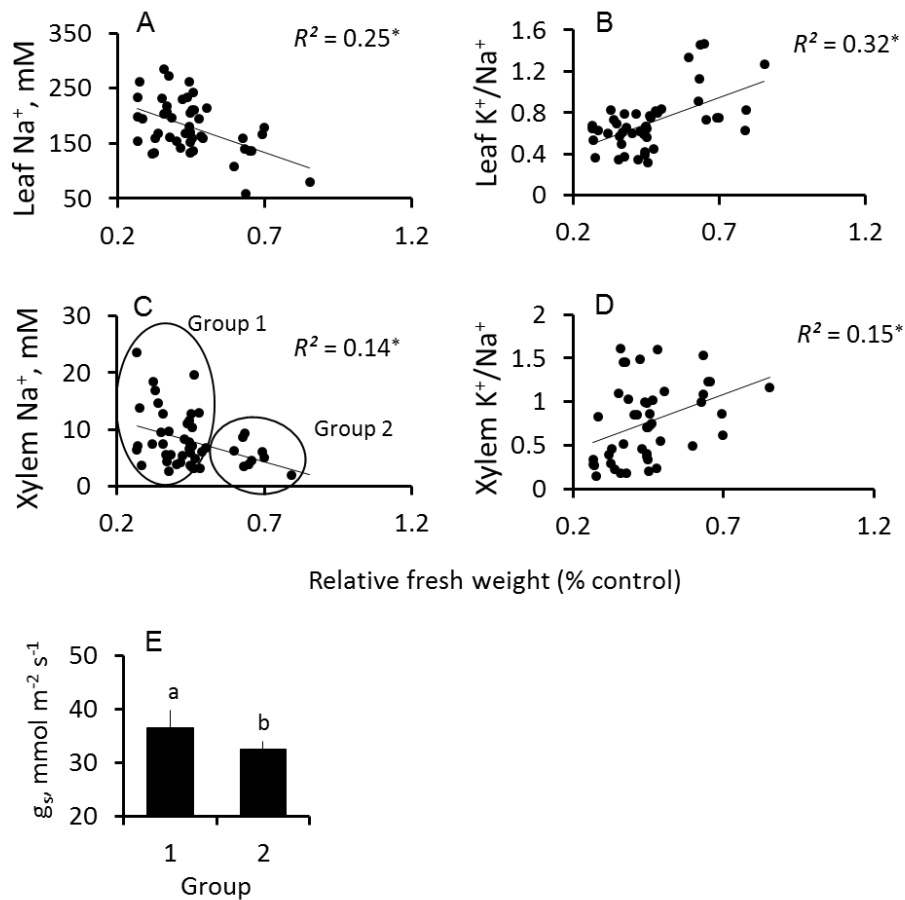
**Fig. 3.** Na<sup>+</sup> concentration in xylem sap (mM) of 46 barley genotypes grown under control and salt stress (200 mM NaCl for 5 weeks) conditions. Mean  $\pm$  SE (n = 6). A – Control; B – salinity; C – relative (folds of control).



**Fig. 4.** K<sup>+</sup> concentration in xylem sap (mM) of 46 barley genotypes grown under control and salt stress (200 mM NaCl for 5 weeks) conditions. Mean  $\pm$  SE (n = 6). A – Control; B – salinity; C – relative (folds of control).

A significant negative correlation ( $R^2 = 0.25$ ;  $P < 0.05$ ) was observed between leaf Na<sup>+</sup> content and relative fresh weight (FW) under saline conditions (Fig. 5A), while K<sup>+</sup>/Na<sup>+</sup> discrimination in leaf under the treatment of 200 mM NaCl in leaf was positively and significantly ( $R^2 = 0.32$ ;  $P < 0.05$ ) correlated with FW (Fig. 5B). Similarly, Na<sup>+</sup> concentration in the xylem sap under salt stress was found to have a significant negative relationship with FW ( $R^2 = 0.14$ ;  $P < 0.05$ ; Fig. 5C). When barley genotypes were clustered into two groups (Group 1 and Group 2; Fig. 5E), the mean value of stomatal conductance ( $g_s$ ) in Group 1 was significantly higher than that in Group 2 ( $P < 0.05$ ). This indicates a potential linkage between the shoot transpiration and xylem Na<sup>+</sup> loading under salinity stress. The ratio of K<sup>+</sup>/Na<sup>+</sup> in xylem sap associated positively and significantly ( $R^2 = 0.15$ ;  $P < 0.05$ ; Fig. 5D) with fresh weight (FW).

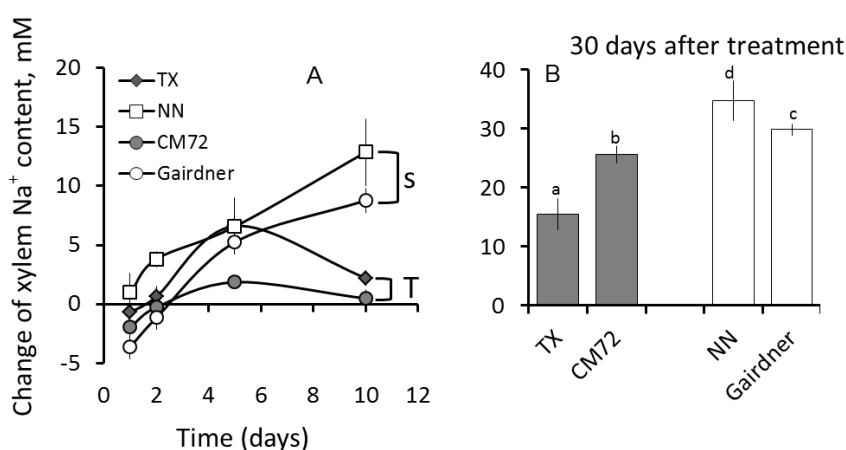




**Fig. 5.** Correlations between relative fresh weight (FW) and leaf Na<sup>+</sup> content (A), leaf K<sup>+</sup>/Na<sup>+</sup> ratio (B), xylem Na<sup>+</sup> content (C) and xylem K<sup>+</sup>/Na<sup>+</sup> ratio (D) in 46 barley genotypes grown under 200 mM NaCl for 5 weeks; the inserted histogram in Panel A is the value of stomatal conductance ( $g_s$ ) of the two groups (1&2; the two groups were clustered according to salinity tolerance – relative fresh weight in C) under saline condition.

The kinetics of xylem Na<sup>+</sup> loading was studied using four contrasting barley genotypes (TX9425 and CM72 – tolerant; Naso Nijo (NN) and Gairdner sensitive) selected from the large screen of 46 barley genotypes using the leaf and xylem ion content as the physiological characterises (Fig. 6). These barley genotypes were chosen, due to their contrasting performance under salinity stress; moreover, they were commonly used in the previous study in our laboratory. Each dot point in Figure 6A is the increment of Na<sup>+</sup> content in xylem

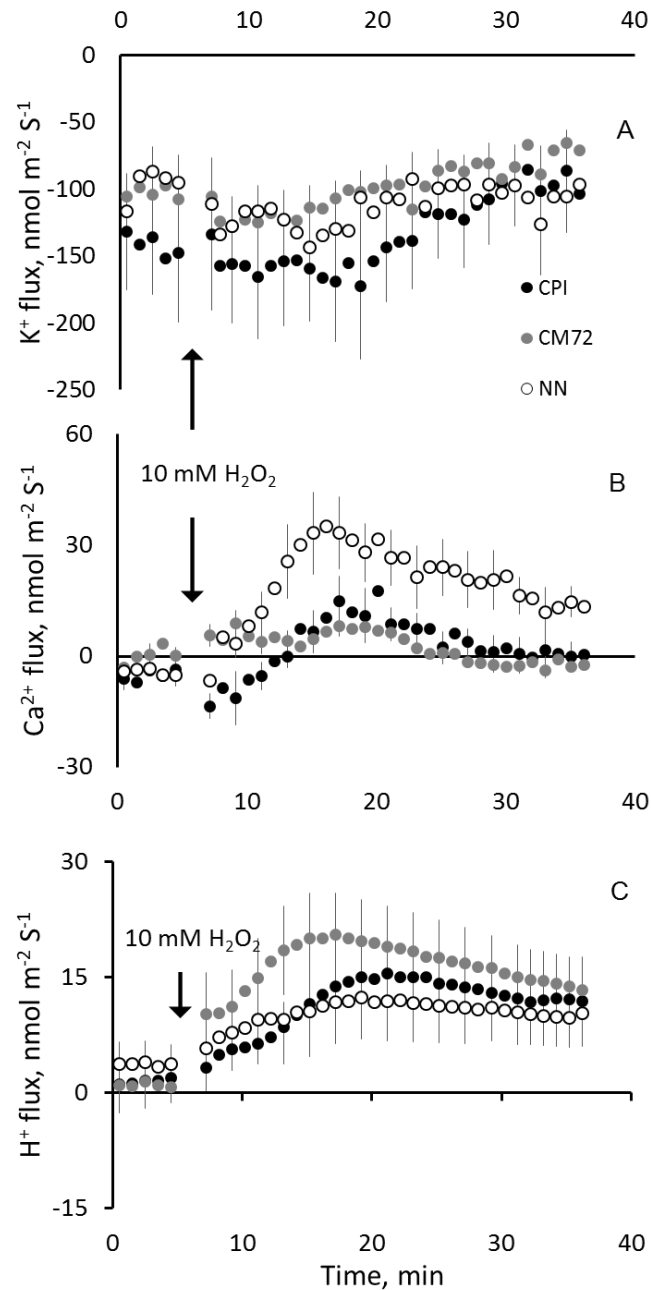
sap compared with time zero (just before the stress applied). Xylem Na<sup>+</sup> content in the salt-tolerant genotypes TX9425 and CM72 increased gradually for the first 5 days upon 200 mM NaCl treatment and then slowed down. However, the salt-sensitive barley cultivars NN and Gairdner showed a gradual and progressive increase in Na<sup>+</sup> accumulation in the xylem. As a result, the xylem sodium content in salt-sensitive genotypes on 10<sup>th</sup> day was approximately 5 folds of the value measured on 1<sup>st</sup> day. Long-term 30 day) salinity treatments exacerbated the difference between tolerant and sensitive genotypes, with 1.5 fold on average higher xylem Na<sup>+</sup> concentrations observed in salt-sensitive cultivars (Fig. 6B).



**Fig. 6.** (A) The kinetics of the increment of xylem Na<sup>+</sup> content in four contrasting barley genotypes (open symbols stand for tolerant genotypes: TX9425 and CM72; shaded symbols stand for sensitive genotypes: Naso Nijo and Gairdner) grown under 200 mM NaCl for 10 days; (B) The xylem sap Na<sup>+</sup> content of these four genotypes grown under 200 mM NaCl for 30 days was measured.

Three genotypes – a wild barley (CPI) and two contrasting genotypes (CM72 – salt tolerant; NN – salt sensitive) were selected for MIFE experiments to investigate the mechanisms and signalling pathway underlying xylem ion loading. Addition of 10 mM H<sub>2</sub>O<sub>2</sub> didn't show any significant impact on K<sup>+</sup> efflux (Fig. 7A). However, both Ca<sup>2+</sup> and H<sup>+</sup> fluxes were strongly responsive to H<sub>2</sub>O<sub>2</sub> treatment and showed some genotypic difference (Fig. 7B and 7C). 10

mM H<sub>2</sub>O<sub>2</sub> treatment resulted in a sharp increase in net Ca<sup>2+</sup> influx into root stele in all the genotypes (Fig. 7B); this uptake was significantly ( $P < 0.05$ ) higher in salt-sensitive NN cultivar (33, 11 and 8 nmol m<sup>-2</sup> s<sup>-1</sup> in NN, CPI and CM72, respectively). 30 min after the application of 10 mM H<sub>2</sub>O<sub>2</sub>, the salt-sensitive genotype (NN) still had significantly ( $P < 0.05$ ) higher Ca<sup>2+</sup> influx than salt-tolerant genotypes. Similarly, H<sup>+</sup> influx reached a peak value 15 min after the addition of 10 mM H<sub>2</sub>O<sub>2</sub> and NN was found to have the highest magnitude of H<sup>+</sup> uptake into a root stele than the other two tolerant genotypes (significant at  $P < 0.5$ ; Fig. 7C).

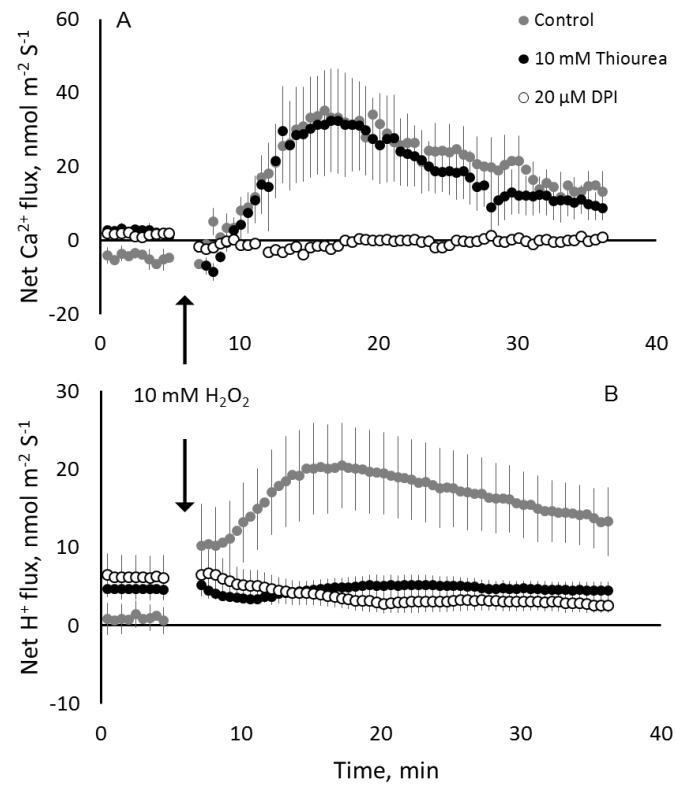


**Fig. 7.** Transient  $\text{K}^+$  (A),  $\text{Ca}^{2+}$  (B) and  $\text{H}^+$  (C) fluxes responses measured from root stele in 3 barleys genotypes (CPI, CM72 and Naso Nijo) in response to acute 10 mM  $\text{H}_2\text{O}_2$  treatment. Mean  $\pm$  SE (n = 6).

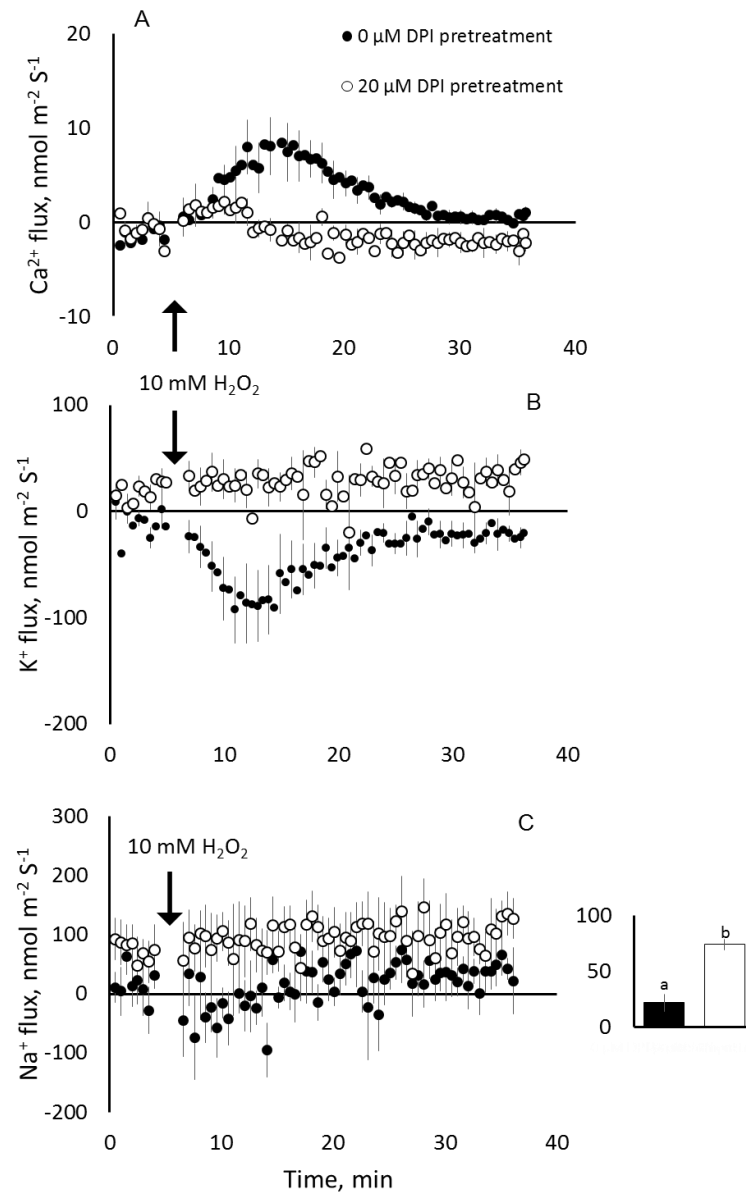
Given the fact that cultivar NN showed highest sensitivity to  $\text{H}_2\text{O}_2$  treatment, this genotype was chosen for the pharmacological experiments. Two chemicals were applied here: thiourea (a hydroxyl radical scavenger; Demidchik *et al.* 2010) and DPI (diphenyleneiodonium chloride, the inhibitor of NADPH-

oxidase; (Wind *et al.* 2008). Transient Ca<sup>2+</sup> and H<sup>+</sup> fluxes were measured from root stele in response to 10 mM H<sub>2</sub>O<sub>2</sub>, the stele that was pre-treated with either 10 mM thiourea or 20 μM DPI for 0.5 h (Fig. 8). There was no Ca<sup>2+</sup> uptake observed in the root stele pre-treated with 20 μM DPI after an acute addition of 10 mM H<sub>2</sub>O<sub>2</sub>, compared with a significant Ca<sup>2+</sup> influx found in both control and 10 mM thiourea pre-treated root stele (Fig. 8A). Root stele pre-treatment with 10 mM thiourea and 20 μM DPI inhibited the uptake of H<sup>+</sup> in response to the application of 10 mM H<sub>2</sub>O<sub>2</sub>, with the observation that control has significantly higher H<sup>+</sup> influx compared with the other two treatments (Fig. 8B).

In another set of experiments, the 3-day old barley seedlings were grown under 100 mM NaCl for 24 h and then ion fluxes were measured after root stele was peeled and put into 20 mM NaCl overnight (to mimic typical xylem Na<sup>+</sup> concentration found in salt-grown plants). Some tissues were incubated in the presence of 20 μM DPI while others were used as controls. 10 mM H<sub>2</sub>O<sub>2</sub> treatment caused transient Ca<sup>2+</sup> flux similar to one observed in Fig. 9A in control specimens, while the pre-treatment with 20 μM DPI reduced Ca<sup>2+</sup> influx (Fig. 9A). In contrast, root stele pre-treated with DPI was observed to have significantly bigger K<sup>+</sup> efflux after the addition of 10 mM H<sub>2</sub>O<sub>2</sub> while the K<sup>+</sup> efflux in control root stele didn't change much after H<sub>2</sub>O<sub>2</sub> application (Fig. 9B). No significant Na<sup>+</sup> flux was measured from either control or DPI-pre-treated root stele after the onset of 10 mM H<sub>2</sub>O<sub>2</sub>.

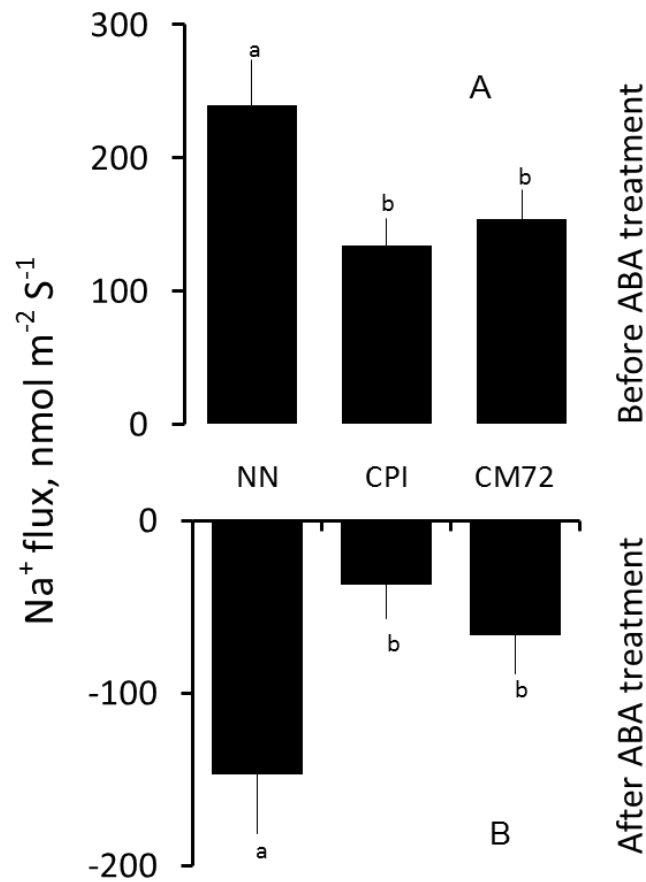


**Fig. 8.** Transient  $\text{Ca}^{2+}$  (A) and  $\text{H}^+$  (B) flux responses measured from root stele in 3 barley genotypes (CPI, CM72 and Naso Nijo; pre-treated with/without 10 mM thiourea and 20  $\mu\text{M}$  DPI for 0.5 h) in response to acute 10 mM  $\text{H}_2\text{O}_2$  treatment. Mean  $\pm$  SE ( $n = 6$ ).



**Fig. 9.** Transient  $\text{Ca}^{2+}$  (A),  $\text{K}^+$  (B) and  $\text{Na}^+$  (C) flux responses measured from root stele in 3 barley genotypes in response to acute 10 mM  $\text{H}_2\text{O}_2$  treatment (CPI, CM72 and Naso Nijo; grown under 100 mM NaCl for 24 h, then the root stele was extracted by peeling and put into BSM containing 20 mM NaCl overnight. Before measuring the ion fluxes, the samples were with/without 20  $\mu\text{M}$  DPI for 0.5 h). Mean  $\pm$  SE ( $n = 6$ ). The inserted histogram in Panel C shows the mean value of  $\text{Na}^+$  flux 30 min after the application of 10 mM  $\text{H}_2\text{O}_2$ .

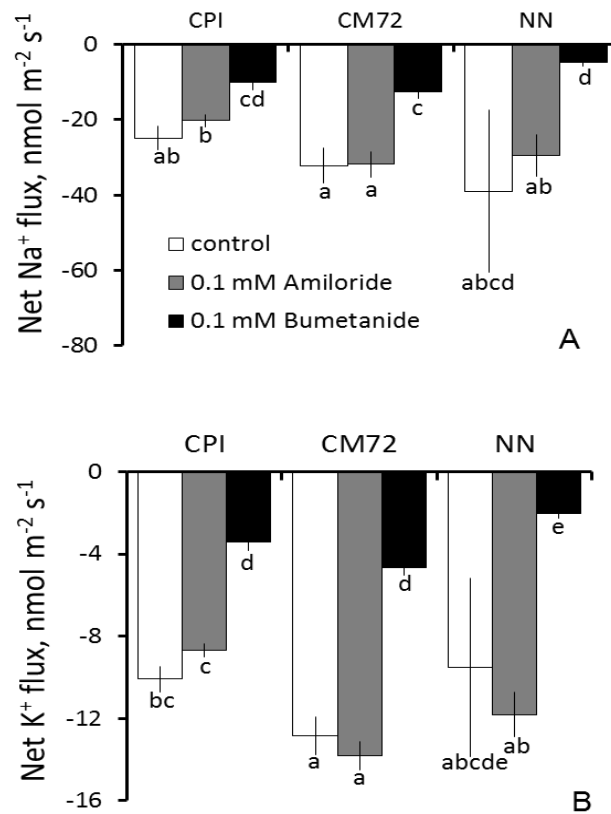
To clarify whether abscisic acid (ABA) is involved in regulation of xylem ion loading, the root stele Na<sup>+</sup> flux was measured in response to acute 10  $\mu$ M ABA treatment. Figure 10A shows the mean values of the Na<sup>+</sup> influx measured on root stele in 3 barley genotypes grown under 200 mM for 24 h. As one can see, NN has significantly higher Na<sup>+</sup> influx into xylem than CPI and CM72. After the application of 10  $\mu$ M ABA, Na<sup>+</sup> influx changed Na<sup>+</sup> efflux from xylem. However, the decrease in NN is significantly higher than the other two salt-tolerant genotypes (Fig. 10B).



**Fig. 10.** Mean Na<sup>+</sup> flux values measured from root stele (3-day seedlings grown under 100 mM NaCl for 24 h) before and after (A and B respectively) the addition of 10  $\mu$ M ABA. Mean  $\pm$  SE (n = 6)



Effect of the inhibitors of SOS1-like-exchanger and chloride-cation-co-transporter (CCC) inhibitor (amiloride and bumetanide, respectively) on the Na<sup>+</sup> and K<sup>+</sup> fluxes in root stele under salinity stress was also studied here (Fig. 11). Pre-treatment of root stele with 0.1 mM amiloride resulted in no significant change in K<sup>+</sup> and Na<sup>+</sup> fluxes in either of three barley genotypes, compared with the ion fluxes measured from root stele without amiloride-pre-treatment. However, the root stele pre-treated with 0.1 mM bumetanide showed some difference in both Na<sup>+</sup> and K<sup>+</sup> efflux (Fig. 11A and 11B). Here Na<sup>+</sup> and K<sup>+</sup> efflux were significantly lower than those in the control root stelar tissue (the value of K<sup>+</sup> efflux is only about 1/3 of the control value and Na<sup>+</sup> efflux is only about 1/4 of the control value). The K<sup>+</sup> and Na<sup>+</sup> efflux in the root stele of NN is significantly lower than the ion flux values in tolerant genotypes (CPI and CM72;  $P < 0.05$ ).



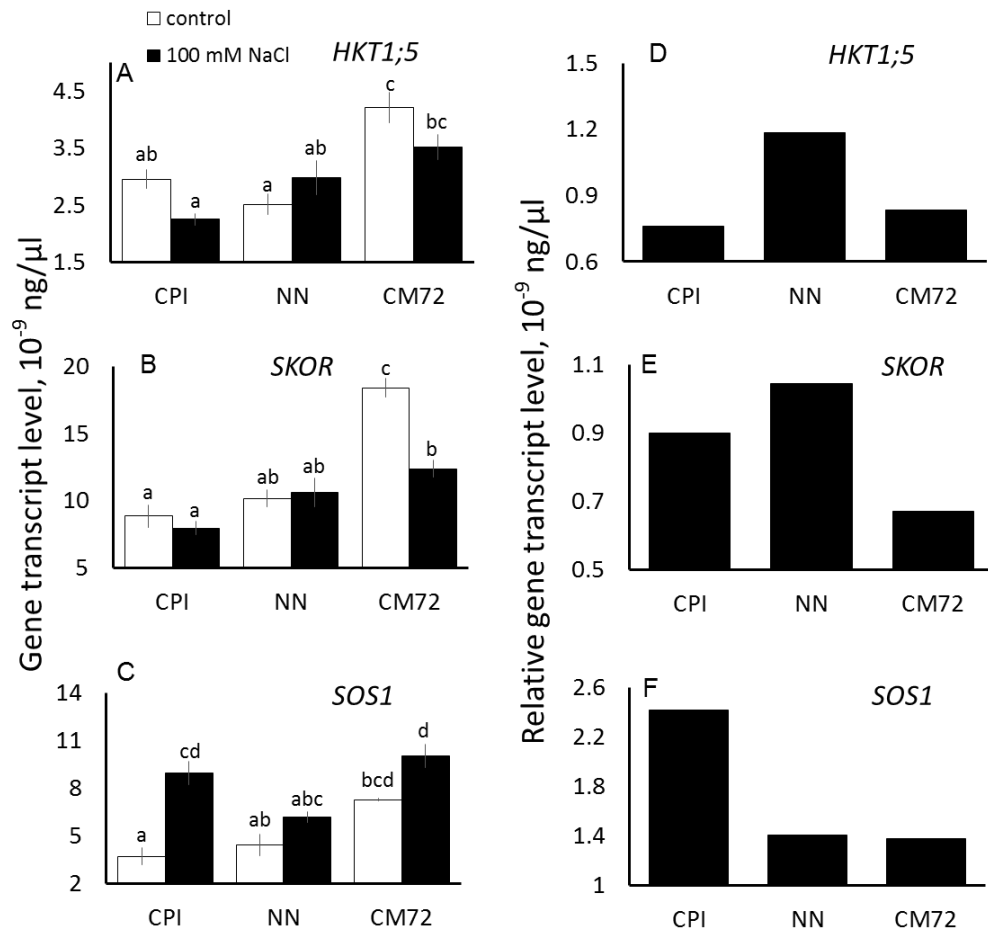
**Fig. 11.** Mean Na<sup>+</sup> (A) and K<sup>+</sup> (B) flux values measured from root stele pre-treated with/without 0.1 mM Amiloride or 0.1 mM Bumetanide, (CPI, CM72 and Naso Nijo; grown under 100 mM NaCl for 24 h, then root stele was peeled and exposed to 20 mM NaCl overnight before the measurement of ion fluxes). Mean  $\pm$  SE (n = 6).

Since all three genes (*SOS1*, *HKT1;5* and *SKOR*) haven't been characterized in barley, BLAST was conducted in barley genome database (Plant Genome and Systems Biology, PGSB) using the known genes from wheat or Arabidopsis (*TdSOS1*, *TaHKT1;5* and *AtSKOR*). The identity rate was 94%, 83% and 73% respectively, and the CDS could be found in Appendix (Table 1). The transcript level of *HvHKT1;5\_like*, *HvSKOR\_like*, *HvSOS1\_like* and *HvGAPdH* genes in root epidermal and stelar tissue in three barley genotypes grown under control and saline conditions were quantified using RT-qPCR. The result of reference gene (*HvGAPdH*) shows that its transcriptional level is the same among different treatments in root stele and epidermis in all the three barley genotypes

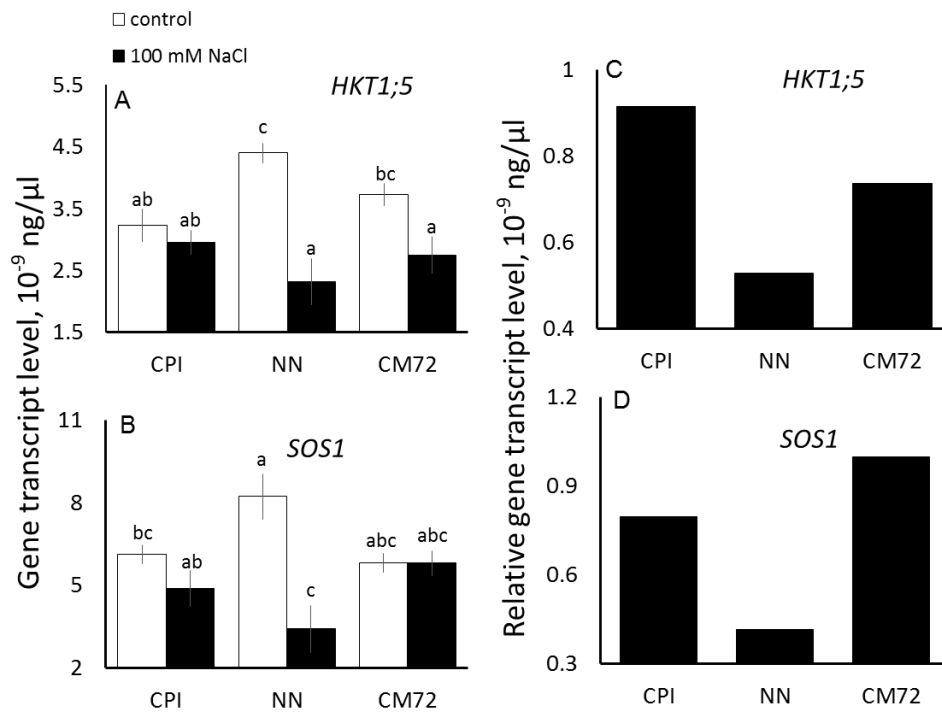
(Table 2). The transcript level of *HvHKT1;5\_like* gene decreased in root stele in the salt-tolerant genotypes after transferred into BSM containing 100 mM NaCl for 24 h (Fig. 12A), while this gene transcript level increased in NN (salt-sensitive genotype, approximately 1.18 folds of control). The same phenomenon was observed in the transcript level of *HvSKOR\_like* which was slightly up-regulated in NN root stele under the treatment with 100 mM NaCl (Fig. 12B; about 1.05 folds of control), while salt-tolerant cultivars were observed to have lower transcriptional level of *HvSKOR\_like* under saline condition (10% and 33% decrease compared with the respective controls CPI and CM72). Unlike the first two genes, *HvSOS1\_like* gene in root stele increased upon 100 mM NaCl treatment compared with the control values in all three genotypes (Fig. 12C). This increase was significantly higher in CPI compared with NN and CM72 (2.4, 1.4 and 1.37 folds of control, respectively). It has been noticed that the absolute transcript level of *HvSOS1\_like* gene in NN under saline condition was significantly lower than that in tolerant genotypes. Transcript level of both *HvHKT1;5\_like* and *HvSOS1\_like* decreased in root epidermal tissue among all three genotypes under salt stress (Fig. 13A and Fig. 13B). NN was found to have significantly lower expression level of these two genes, compared with CPI and CM72.

**Table 2** Transcript level of *HvGAPdH2* gene expression ( $10^{-8}$  ng/ $\mu$ l) in root epidermis and stelar in three barley genotypes (3-day-old seedlings grown under 100 mM NaCl for 24 h)

	Genotypes	Control	100 mM NaCl
Epidermis	CPI	2.64 $\pm$ 0.08	2.44 $\pm$ 0.19
	NN	2.46 $\pm$ 0.07	2.61 $\pm$ 0.48
	CM72	2.46 $\pm$ 0.10	2.76 $\pm$ 0.16
Stele	CPI	2.27 $\pm$ 0.21	2.34 $\pm$ 0.11
	NN	2.35 $\pm$ 0.19	2.64 $\pm$ 0.26
	CM72	2.82 $\pm$ 0.16	2.95 $\pm$ 0.24



**Fig. 12.** Transcript level of *HvHKT1;5\_like* (A), *HvSKOR\_like* (B) and *HvSOS1\_like* (C) gene expression in root stele in three barley genotypes (3-day-old seedlings grown under 100 mM NaCl for 24 h). D, E and F are the relative transcript levels of these three genes respectively (the value under saline condition/the value under control condition). Mean  $\pm$  SE (n = 6).



**Fig. 13.** Transcript level of *HvHKT1;5*<sub>like</sub> (A) and *HvSOS1*<sub>like</sub> (B) gene expression in root epidermis in three barley genotypes (3-day-old seedlings grown under 100 mM NaCl for 24 h). C and D are the relative transcript levels of these two genes respectively (the value under saline condition/the value under control condition). Mean ± SE (n = 6).

## Discussion

### *Low shoot Na<sup>+</sup> concentration and K<sup>+</sup>/Na<sup>+</sup> discrimination are important for salinity tolerance in barley under mild stress*

Both leaf and xylem Na<sup>+</sup> content correlate negatively with FW under the long-term moderate salt stress (200 mM NaCl for 5 weeks; Fig. 5A & 5B) supporting the notion (Tester and Davenport 2003; Munns and Tester 2008) that reducing Na<sup>+</sup> loading into xylem or retrieval of Na<sup>+</sup> from xylem is one of the critical mechanisms contributing to salinity tolerance in plants. Also, K<sup>+</sup>/Na<sup>+</sup> ratio in both leaf sap and the xylem correlates positively with FW under saline condition (Fig. 5B & 5D), but no such correlation was observed for K<sup>+</sup> content

in these tissues. This suggests that it is Na<sup>+</sup>, not K<sup>+</sup>, which determines the correlation between K<sup>+</sup>/Na<sup>+</sup> ratio and FW. This conclusion is in contrast to some previous results from our laboratory (e.g. Shabala *et al.* 2010) showing that restricting Na<sup>+</sup> loading into the xylem was not essential for conferring salinity tolerance in barley under saline condition. However, it should be noted that the latter study used extremely high (320 mM NaCl) salinity treatment while in this work barley plants were treated with only 200 mM NaCl. Thus, it is plausible to suggest that the essentiality of Na<sup>+</sup> exclusion may differ depending on severity of the stress. Following this notion, plants exposed to 200 mM NaCl will probably have to deal mainly with osmotic component of salt stress, while more severe 320 NaCl mM treatment (as in Shabala *et al.* 2010) would require higher tissue tolerance which will be determined mostly by plant's ability to sequester Na<sup>+</sup> in mesophyll cell vacuoles that is largely dependent on the extent of Na<sup>+</sup> accumulation in the shoot (Reviewed by Colmer *et al.* 2003; Munns and Tester 2008; Shabala 2013). This capacity is not important for mild 200 mM treatment; to adjust to the latter conditions plants ideally need to adjust rapidly their shoot osmotic potential by sending appropriate amounts of Na<sup>+</sup> to the shoot within the first few days (Fig. 6) and shut down any further Na<sup>+</sup> delivery to the shoot. However, not all cultivars are efficient in arresting further xylem Na<sup>+</sup> loading. Also, Na<sup>+</sup> delivery to the shoot was positively correlated with 'transpiration pull' (g<sub>s</sub> values; insert in Fig. 5C), reflecting a well-known trade-off between yield and stress tolerance.

### ***Salt-tolerant genotypes can utilize Na<sup>+</sup> more efficiently in both short and long term under moderate salinity***

As commented above, excessive Na<sup>+</sup> could be delivered from root to shoot via xylem vessels, for the purpose of osmotic adjustment occurring at the beginning of salinity stress. Here, we confirmed this hypothesis, not only at plant physiological level (Fig. 6), but also at transcriptional level (Fig 12 and 13). Both tolerant cultivars (CPI and CM72) down-regulated *HKT1;5*-like gene expression in the stele while salt-sensitive genotype NN increased *HKT1;5* transcript levels (Fig. 12A). As *HKT1;5* major role is Na<sup>+</sup> retrieval from the xylem (Byrt *et al.* 2007; James *et al.* 2011; Byrt *et al.* 2014), this should result

in a lower rate of Na<sup>+</sup> loading into the xylem in sensitive genotypes which was indeed the case for the first several days (Fig. 6). At the same time, *HvSOS1\_like* gene transcripts were significantly up-regulated, with the strongest effect observed in the most tolerant CPI genotype (Fig. 12C). This is fully consistent with the suggested role of SOS1 in xylem loading (Shi *et al.* 2002; Shi *et al.* 2003; Feki *et al.* 2014; also see previous chapter). So, the most tolerant CPI plant (Fig. 12) showed up-regulation of *SOS1* and down-regulation of *HKT1;5* maximising Na<sup>+</sup> loading into xylem in the first days after stress onset. On the contrary, the most sensitive NN genotype maximised Na<sup>+</sup> retrieval from the xylem increasing substantially *HKT1;5* expression and having the lowest gain in *SOS1* transcripts in the stele. Again, it is all highly consistent with physiological data reported in Fig. 6. Overall, rapid delivery of Na<sup>+</sup> to the shoot in tolerant varieties could allow these genotypes to use it as a cheap osmolyte avoiding yield penalty due to high carbon cost of organic osmolytes production (Munns and Gilliham 2015).

Another interesting phenomenon were observed changes in *HKT1;5* and *SOS1* transcript changes in root epidermis (Fig. 13). The biggest alterations were reported for salt-sensitive genotype NN. It may be suggested that decreased expression of *SOS1* in root epidermis in this case may be used as an optimal strategy adopted by plant to retain more Na<sup>+</sup> in the root (rather than pump it out) to compensate for its inability to load Na<sup>+</sup> into the xylem. However, this strategy may be highly detrimental, as a failure to actively pump Na<sup>+</sup> out by the plasma membrane SOS1 Na<sup>+</sup>/H<sup>+</sup> exchanger (Shi *et al.* 2002) may jeopardise plant's ability to cope with Na<sup>+</sup> load in long-term.

### ***NADPH oxidase-mediated apoplastic H<sub>2</sub>O<sub>2</sub> production acts upstream of xylem Na<sup>+</sup> loading and is causally related to ROS-inducible Ca<sup>2+</sup> uptake systems in the xylem parenchyma tissue***

Hydrogen peroxide is one of the known signalling molecules that has an ability to travel long distances (Baxter *et al.* 2014; Gilroy *et al.* 2014) and enables communication between remote plant tissue and organs (Mittler and Blumwald 2015). Moreover, propagating H<sub>2</sub>O<sub>2</sub> waves seem to be intrinsically

integrated with propagating Ca<sup>2+</sup> signals (Steinhorst and Kudla 2013; Gilroy *et al.* 2014). Importantly, H<sub>2</sub>O<sub>2</sub> levels increase dramatically in stressed plant tissues (Miller *et al.* 2010), specifically under saline conditions (Bose *et al.* 2014). In this work we have mimicked this increase by exogenously applying physiologically-relevant amounts of H<sub>2</sub>O<sub>2</sub> to stelar tissues, in order to investigate its role as a potential regulator of xylem ion loading.

In a full agreement with the above notions, H<sub>2</sub>O<sub>2</sub> treatment induced strong transient Ca<sup>2+</sup> uptake into the stele (Fig. 7) which was the strongest in salt-sensitive variety NN. Previous studies (Demidchik and Maathuis 2007) have implicated NADPH oxidase as a major source of apoplastic ROS production and the upstream component of net Ca<sup>2+</sup> uptake in root epidermis. Here we show that inhibition of NADPH oxidase by DPI was significantly reduced, reflected in the magnitude of H<sub>2</sub>O<sub>2</sub>-induced Ca<sup>2+</sup> ion fluxes measured from root stele (Fig. 8 & 9). This is fully consistent with the idea of NADPH oxidase acting upstream of Ca<sup>2+</sup> signalling in stelar tissues in both control (Fig. 8A) and salt-treated plants (Fig. 9A). NADPH oxidase is known to be activated by cytosolic Ca<sup>2+</sup> (Lecourieux *et al.* 2006), forming a positive feedback loop. The fact that the sensitivity of stelar tissues to hydrogen peroxide was highest in salt-sensitive NN genotype (Fig. 7) may be therefore indicative of a possibility of uncontrollable increase in tissue ROS production under stress conditions. Given the fact that many plasma (NSCC and KOR) and tonoplast-based (NHX, PPase, V-PPase) membrane transporters are activated by ROS (and, specifically, by H<sub>2</sub>O<sub>2</sub>), this might lead to major alterations in intracellular ionic homeostasis and explain poor plant performance of NN genotype under saline conditions.

Further supporting evidence for this model can be found in Figure 9B showing that inhibition of NADPH oxidase by DPI had a major effect on the process of K<sup>+</sup> loading into xylem vessels, with the observation of net K<sup>+</sup> influx into the stele (e.g. prevention of xylem K<sup>+</sup> loading) following inactivation of NADPH oxidase. This process could be mediated by SKOR, a stelar K<sup>+</sup> outward rectifier (Véry *et al.* 2014) which is known to be expressed at the xylem parenchyma interface (Wegner and De Boer 1997) and mediate potassium delivery to the shoot. It also appears that NADPH oxidase-mediated apoplastic



H<sub>2</sub>O<sub>2</sub> production acts upstream of xylem Na<sup>+</sup> loading, as inhibition of NADPH oxidase by DPI has also resulted in reduced net Na<sup>+</sup> influx into stele (e.g. reduced Na<sup>+</sup> retrieval from the xylem; Fig. 9).

### *ABA down-regulates Na<sup>+</sup> retrieval from xylem in barley*

The inhibition of channel-mediated efflux of K<sup>+</sup> and Cl<sup>-</sup> into the xylem by ABA has been well documented using both radioactive and electrophysiological techniques (Cram and Pitman 1972; Roberts 1998; Gilliam 2002). However, the control of xylem Na<sup>+</sup> loading by ABA has been studied much less. Here we report that the application of exogenous ABA could attenuate the activity of retrieval of Na<sup>+</sup> from xylem. Indeed, net Na<sup>+</sup> uptake into the stelar tissue (Fig. 10A) has turned into net Na<sup>+</sup> efflux (e.g. xylem loading) after ABA addition (Fig. 10B). The possible reason for that may be the fact that addition of ABA stimulated H<sup>+</sup> extrusion into xylem (Clarkson and Hanson 1986), affecting activity of SOS1 Na<sup>+</sup>/H<sup>+</sup> antiporter at the plasma membrane of xylem parenchyma cells. Observed net Na<sup>+</sup> efflux from the stele upon ABA treatment (Fig. 10B) is in a good agreement with this model.

### *CCC transporter plays a role in the xylem Na<sup>+</sup> loading in barley*

AtCCC is preferentially expressed in the root and shoot vasculature at the xylem/symplast boundary (Colemoro-Flores *et al.* 2007) and was found to catalyse the co-ordinated symport of K<sup>+</sup>, Na<sup>+</sup>, and Cl<sup>-</sup> into the xylem in *A. thaliana*. In mammalian systems, activity of CCC transporter may be inhibited by application of bumetanide (Starremans *et al.* 2003). In this study, the application of bumetanide reduced both Na<sup>+</sup> and K<sup>+</sup> efflux from xylem vessels (Fig. 11), while amiloride (a known inhibitor of Na<sup>+</sup>/H<sup>+</sup> exchanger; Blumwald and Poole 1985; Kleyman and Cragoe 1988; Cuin *et al.* 2011) only observed little effects on net Na<sup>+</sup> fluxes from stelar tissue (Fig. 11A). This is in contrast to the previous reports of the strong inhibitory effects of amiloride on net Na<sup>+</sup> fluxes in root epidermal cells in wheat (Cuin *et al.* 2011), barley (Schulze *et al.* 2012) and Arabidopsis (Essah 2000) and is suggestive that CCC transporter is

present at the barley root xylem parenchyma and may play a major role in loading both Na<sup>+</sup> and K<sup>+</sup> into the xylem.

### Appendix

**Table 1** CDS of *HvSOS1\_like*, *HvHKT1;5\_like* and *HvSKOR\_like*

CDS of <i>HvSOS1_like</i>
ATGCTGAACAAGGCATTGGAGGCTTTTGGTGATCTCAGGGATGA TGAGGAACCTTGGGCCTGTTGACTGGGTAAATGTGAAGAAATATA TCACGTGTTTGAATAACTTGAAGATGAACAAGCACATCCCCAT GATGTTCCCTGACAAGGATGATCACGTGCATACCTTGAATTTGAA GGATACTCGAGTGCGGCTTTTGAATGGTGTGCAAGCTGCTTACTG GGGAATGCTTGAAGAGGGACGAATAACTCAATCTACAGCAAATA TTTAAATGAGATCAGTTGATGAAGCTATGGATCTTGTCTCTTCCC AATCATTATGTGATTGGAAGGGTTTGCGGTCCAATGTTCAATTCC CAAATTACTATAGGTTCCCTTCAGATGAGCAGGTTGCCACGAAGG CTAGTCACATACTTCACAGTAGAAAGATTGGAGCTAGGATGTTA CATCTGTGCAGCATTTCTCCGTGCTCACAGAATTGCGAGGAGAC AACTACATGATTTTCTTGGTGATAGTGAGATTGCAAGAATTGTCA TCGATGAAAGTACTGCTGCTGGGGAGGAAGCTAAAAAGTTTCTG GAAGACGTTTCGTGTTACATTCCCTCAGGTGCTTCGTGCATTGAAG ACTCGACAAGTAACATATGCGGTATTGACACACTTGAGTGAGTA TATTCAAGACCTTGGGAAGACTGGGTTGCTCGAGGAAAAGGAAA TAGTCCATCTCGATGATGCTTTGCAGAGAGACTTGAAGAAGCTT AAGAGGAATCCGCCACTGGTGAAAATGCCAAGAGTTCACGAACT TCTAAATACCCATCCTTTAGTTGGTGCACTGCCTGCTGCTCTTCGT GATCCATTGTAAAGTAATACAAAGGAAACAATAAAAGGGCATGG AACAGTTCTTTATGCAGAAGGCTCAAGGCCGACTGGTGTATGGC TTGTTTCGAGTGGAATAGTAAAGTGGACAAGTCAGAGACTATGC ACGAGGCATTCATTGGATCCAATTTTGTACATGGAAGCACTTTG GGTCTATATGAGGCATTAATTGGAAAGCCTTATATTTGTGACATT ATTACAGAATCAGTGGTGCATTGTTTCTTCATTGAGGCTGAAAAA

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GCTCAGGTGGCCTGAGAGTTTCCGGAGATCCAGAGGGCCTGGAA  
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CAATGCAATGAACAGAAAGTCTGCTCCTGCTCCAGCTCCAGCTC  
CAGTTCATGCACTTGCTCCTTTTCCACCCCTAGCAGCAGGCCGGC  
AACGCAGGGAGATAGCGGAAGATGACAACTCGAGCGACGAGTC  
TGTGGAGGAAGAAGTTATCATCAGAGTGGACTCTCCCAGCATGC  
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**CDS of *HvHKT1;5* like**

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ATGCACATCCATCCCCAAGTCCCAATCCCCAACACGTCCACAC  
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AACAATGTGGTGTCCCTCATGCGCTCGCTCTCCGGGGTCATACGCG  
CAGCACCACGTCAAGGAGCGCGTGCGCGGTGGCGCCGCGCGCT  
CGCCGGGCGGTTATCGCCGCGCCTCGGCTCGCTGCTCGTCCACGT  
CGCCTACTTCCTCGCCGTCTCCTGGCTCGGTTACCTCGTCCTCGC  
GCAGCTCAGGTTCGCGCGCCGGCGGCGAGGGCACGAGGCGGCCA  
GGCGCCATCGACCTGTTCTTACCGCCGTCTCGGCCGCGACGGTG

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TCGAGCATGTCCACCGTCGAGATGGAGGCGTTCTCCAACGGCCA  
GCTCCTCGTCCTGACCGTGCTCATGTTTCGTCGGCGGCGAGGTGTT  
CTTGTCGCTCCTAGGCCTCGCGTCCAAGTGGTCCAGGCTGAGGA  
AGCAAGCTGTAAGCAAATCCCGGCGCGTCGAGATCCACCACGTC  
GCCGAGCTTGAGATGCCGCCGGTCGCCGCTGCTGGCGAATTCTGA  
CAACCCAACGTCAATGACGTCGACCGCCGAGGAAGAGGTGAGC  
AAGCCGTTGGACCGCTTCGATGACACGAGGCTGCGGCGCGACGC  
GGTGCTGTGCTCTTCTTCGTCGTGCTCGCCATCCTCCTGACGGT  
GCACGTCCTCGGCGCCGCCCATCGTGGCGTACATCTTCCACGC  
GTCGTCTTCGGCGAGGCGGACGCTGCGGAAGAAGGCCCTGAACG  
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GGCGATGCGCGATGCTGGCGGCGACGGTGGCCGGGCTCGTCGCC  
GTGCAGGTGGCGATGTTGTGCGGCATGGAGTGGGGCGGCGCGCT  
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TCCACCCTGGCGCCGGCCATCCTCGTCCTCTTCGTGCTCATGATG  
TACCTGCCTCCATACACGACATGGTTTCCATTTGGAGAGAGCTCG  
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GCGCTTATGGAAATGTGGGCTTCTCCATGGGCTACAGCTGCAGT  
AGGCAGATCAGCCCGGACCGGCTGTGCACTGACAGGTGGACCGG  
CTTTGCTGGGAGGTGGAGCGATTCCGGCAAAGTCATCCTCATTCT  
TGTCATGCTCTTCGGGAGGCTGAAAAAGTTCAGCATGAGCGCGG  
GCAAAGCCTGGAAGCTAAGT

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CDS of *HvSKOR\_like*

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ATGGCGAGGGGGGCAAGGTCGAGGAGGGCGCCGGCGGGGGAGG  
AGGAGGCAGAGAAGGAGTACGAGGTGGACGTGGAGCCCGACCG  
GTGGAAGTCGTCGTCGTCGTCGCGGAGCAGCCGGCTCGAGCTCA  
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GCCGACGGCGACGGGGAGGGCGGGGCTCCTCCGCGGCCTCGTCAT  
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CCTTGGTTGCTTCCCATGGGATGCCATCTATAAGGCTTCTGGACG  
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CGTGTGAATTACCTGTTTACAAGAATAGTGAACTCATACTTGTG  
GAGCTCTATTGTACACACACAGCAGCTTGTATCTTCTATTACCTG  
GCAACAACGCTTCCTGAATCAGTGGAGGGATATACGTGGATCGG  
GAGTTTGCAGCTAGGAGACTATAGCTATTCCCATTTTCAGGGAGA  
TCGATCTTGCCACGCGTTATGTTACATCAATGTACTTTGCCATTG  
TCACCATGGCAACTGTTGGTTACGGTGACATTCATGCTGTAAATG  
TTAGGGAAATGATATTTGTCATGATATATGTTTCCTTTGATATGG  
TTCTTGGAGCTTACCTCATCGGTAACATGACAGCAATGATTGTGA  
AAGGCTCGGCAACCGAGCGATTTCAGGGATAAAATGAAAGAAGT  
TATCAGGTATATGAACAGAAATAGACTTGGAAAGGACATCAGAG  
AACAGATCAAGGGGCATTTGAGGTTGCAGTATGAGAGCAGCTGC  
ACTGAAGCCTCTGTACTTCGGGATATTCCTGTTTCTATTCGTGCA  
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ATTGTTCAAAGGATGTTTACAAGAATTCTTCAACAGATTGTGAT  
TAGGCTGCAAGAAGAGTTCTTCTTACCAGAAGAAGTTATTCTGG  
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TCTAATGTTGGAGCAAGGGAATTCTTTTGGAGAAATTGCTATTCT

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AGTAAATAATGCCGCTTTTTATGGTGACCTTCATCAGTTGACTGG  
CTTAATCCGAGCTGGAGCCAATCCAAAGAACAGTGATTACGATG  
GGCGATCTCCTTTGCATCTAGCAGCTTGCAGAGGGGTATGAAGAT  
GTTGTGCGATTTCCTTATCCATGAAGGGGCTGATATCAATCTAACT  
GATAAATTTGGGAACACACCCTTACTGGAGGCAGTGAGGCAGGG  
GCATGGTAGAGTGGCGTCAATACTCTTCAGCAAAGGAGCCAAGC  
TGAACCTAGAGAATGCCGGTAGCCATCTCTGCATAGCAGTATCA  
AAGGGAGACTCTGATTTTCATTCAAGGGGCTCTAGCTTATGGTGCC  
GATCCAAACTCAAAAGACTACGACCATCGCAATCCTCTCCACAT  
AGCCGCTGCAGAGGGCTTGTATATTATGGCGAAGTTGCTGGTAG  
ACGCTGGTGCAAGTGTGTTTGCAATGGACAGACGCGGTACTACT  
CCTCTGGACGAAGGGCACAAGTCTGGGAGTAAGCCCCTTATAAT  
GCTTCTGGAACAAGCGAAAGCCGACGAGCTCTCCAAGTTCCCCA  
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AGAGGGGGTGATGCTGTGGATTTCCTCGCACTATCAATGAACTCA  
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CGCTTGCTCTGTCAGGATGGCGCGGCGGTCCAGGATGTGGACAT  
GATCAATGACGGGCAGAAGATCTATCTGGTTGGAGACAAGGACA  
CCGGTCATAGCAAG

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## **Chapter 5: *Nax* loci affect *SOS1*-like $\text{Na}^+/\text{H}^+$ exchanger expression and activity in wheat<sup>4</sup>**

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### **Abstract**

Salinity stress tolerance in durum wheat is strongly associated with plant's ability to control  $\text{Na}^+$  delivery to the shoot. Two loci, termed *Nax1* and *Nax2*, were recently identified as being critical for this process and the *HKT1;4* and *HKT1;5* transporters from HKT gene family, respectively, were identified as the candidate genes. At the functional level these transporters are assumed to actively retrieve  $\text{Na}^+$  from the xylem thus limiting the rates of  $\text{Na}^+$  transport from roots to shoots. In this work we show that *Nax* loci also affect activity and expression levels of *SOS1*-like  $\text{Na}^+/\text{H}^+$  exchanger in both root cortical and stelar tissues. Net  $\text{Na}^+$  efflux measured from salt-treated stele by non-invasive ion flux measuring MIFE technique declined in the following sequence Tamaroi (parental line) > *Nax1* = *Nax2* > *Nax1:Nax2* lines. This efflux was sensitive to amiloride (a known inhibitor of  $\text{Na}^+/\text{H}^+$  exchanger) and was mirrored by net  $\text{H}^+$  flux changes. TdSOS1 relative transcript levels were 6 to 10 fold lower in *Nax* lines compared with Tamaroi. Thus, it appears that *Nax* loci confer two highly complementary mechanisms, both contributing to reducing xylem  $\text{Na}^+$  content.

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One of them is enhanced retrieval of  $\text{Na}^+$  back into the root stele via HKT, and another one is a reduced rate of  $\text{Na}^+$  loading into the xylem via SOS1. It is suggested that such duality may play important adaptive role by providing plant with a greater versatility to respond to changing environment and control  $\text{Na}^+$  delivery to the shoot.

**Key words:** salinity stress; xylem loading; sodium; potassium

### Introduction

Soil salinity severely affects plant growth and limits agricultural crop production (Qadir *et al.* 2014; Shabala *et al.* 2014). Approximately 20% of the world's cultivated land, which accounts for over 6% of the world total area, is currently threatened by salinity (FAO 2008). Elevated salt concentration in soil results in accumulation of the toxic concentration of  $\text{Na}^+$  in leaves (Munns and Tester 2008; Rahnema *et al.* 2010). Therefore, control of  $\text{Na}^+$  long-distance transport and the ability of retrieving  $\text{Na}^+$  from the xylem are considered amongst most essential traits conferring salinity tolerance (Munns and Tester 2008; Shabala *et al.* 2013).

Wheat is one of the most important cereal crops worldwide that provides approximately one fifth of the total calorific input of the world's population (FAO 2010). Among wheat varieties, durum (pasta) wheat (*Triticum turgidum* L. subsp. *durum*) is generally less tolerant to salt stress than bread wheat (*Triticum aestivum*; Munns *et al.* 2006; Munns and Tester 2008), mainly because of high rates of  $\text{Na}^+$  accumulation and poor  $\text{K}^+/\text{Na}^+$  discrimination (Gorham *et al.* 1990; Munns and James 2003; Chen *et al.* 2007; Genc *et al.* 2007). To improve salt tolerance of durum wheat, two loci that conferred reduced  $\text{Na}^+$  accumulation in the shoot, termed *Nax1* and *Nax 2*, were found in an unusual genotype named Line 149 (Munns and James 2003). Lines containing either *Nax1* or *Nax2* loci were observed having lower rates of  $\text{Na}^+$  transport from roots to shoots, due to the lower rate of net  $\text{Na}^+$  loading into the xylem (James *et al.* 2006). Both genes can unload  $\text{Na}^+$  from the xylem in the root, while *Nax1*

can also unload  $\text{Na}^+$  from the xylem in the leaf base, the sheath, leading to a high ratio of  $\text{Na}^+$  concentration between sheath and blade (James *et al.* 2006). Lines with *Nax1* or *Nax2* also had higher rates of  $\text{K}^+$  transport from root to shoot, resulting in an enhanced discrimination of  $\text{K}^+$  over  $\text{Na}^+$  (James *et al.* 2006). *Nax1* was indentified by fine mapping as a  $\text{Na}^+$  transporter of the *HKT* gene family *TmHKT1;4* (Huang *et al.* 2006), while *Nax2* was located on chromosome 5A and identified as *TmHKT1;5* (Byrt *et al.* 2007). It was localised on the plasma membrane of cells surrounding the xylem, and was found to confer a yield benefit of 25% on saline soil (Munns *et al.* 2012). A recent study found that a closely related gene, *TaHKT1;5-D* is also localised on the plasma membrane in wheat root stele and operates in retrieving  $\text{Na}^+$  from the xylem vessels in root thus restricting the transport of  $\text{Na}^+$  from the root to the leaves in bread wheat (Byrt *et al.* 2014).

While  $\text{Na}^+$  retrieval from the xylem before it reaches the sensitive photosynthetic tissues is indeed essential for plant performance under saline conditions, the same effect may be achieved by reducing amounts of  $\text{Na}^+$  loaded into the xylem. It was argued (Shabala 2003; Shabala *et al.* 2013) that the ideal scenario for a plant would be to quickly send the amount of  $\text{Na}^+$  to the shoot required to rapidly achieve full osmotic adjustment and maintain the normal growth rate (hence, no yield penalties). Once this is achieved, it would be advantageous for a plant to reduce the rate of xylem  $\text{Na}^+$  loading to the absolute minimum required for driving cell turgor in newly growing tissues but, at the same time, preventing any excessive Na being accumulated in photosynthetic active leaf tissues. This poses a question about the molecular nature of the mechanisms mediating xylem  $\text{Na}^+$  loading, and the modes of their control. Recent thermodynamic analysis showed that channel-mediated xylem  $\text{Na}^+$  loading might dominate at the early stages of salt stress (minutes to hours) while longer exposures to salinity (hours and days) will require thermodynamically active xylem  $\text{Na}^+$  loading (Shabala *et al.* 1997). Two possible candidates were proposed. One is a SOS1  $\text{Na}^+/\text{H}^+$  exchanger (Shi *et al.* 2002), and another one is cation-Cl (CCC) co-transporter (Colmenero-Flores *et al.* 1999).



The SOS signal pathway has been regarded as a key mechanism in maintaining intracellular ion homeostasis under saline conditions (Zhu *et al.* 1998; Hasegawa *et al.* 2000; Sanders *et al.* 2000; Zhu 2001). According to the current view, elevated  $\text{Na}^+$  causes changes in cytosolic free  $\text{Ca}^{2+}$  (Knight *et al.* 1997), which can be sensed by SOS3, a myristoylated  $\text{Ca}^{2+}$ -binding protein (Liu and Zhu 1998; Ishitani *et al.* 2000). SOS3 interacts with and activates SOS2 (a serin/threonine proterin kinase), forming a SOS2/SOS3 complex (Halfter *et al.* 2000; Liu *et al.* 2000). This complex can regulate the expression level of the *SOS1* gene. *AtSOS1* has been identified as  $\text{Na}^+/\text{H}^+$  antiporters localised in epidermal cells at the root tip and also in parenchyma cells at the xylem/symplast boundary of roots, stems and leaves, which control long-distance  $\text{Na}^+$  transport in plants (Shi *et al.* 2002). Overexpression of SOS1 in transgenic *Arabidopsis* has been shown to improve salt tolerance (Shi *et al.* 2003). Feki *et al.* have identified the *TdSOS1* gene from durum wheat, and the heterologous expression of *TdSOS1* in a yeast strain lacking endogenous  $\text{Na}^+$  efflux proteins showed complementation involving cation efflux (Feki *et al.* 2011). Expression of a truncated form of wheat *TdSOS1* in *Arabidopsis sos1-1* mutant exhibited an improved salt tolerance (Feki *et al.* 2014).

In this study, we report we used *Nax1* and *Nax2* durum wheat lines to provide the supporting evidence for the role of SOS1-mediated  $\text{Na}^+$  loading into the xylem in this species. We have tested the hypothesis that reduced  $\text{Na}^+$  accumulation in the shoot of *Nax* lines could be conferred not only by higher  $\text{Na}^+$  retrieval from but also by reduced  $\text{Na}^+$  loading into the xylem. Our electrophysiological and molecular data fully support this hypothesis and suggest that *Nax* loci regulates activity and expression levels of SOS1-like  $\text{Na}^+/\text{H}^+$  exchanger in wheat, and that down-regulation of this transporter in *Nax* lines improves plant performance under saline conditions. This mechanism operates in addition, or instead of, reported increased  $\text{Na}^+$  retrieval from the xylem by HKT transporter, thus reducing the overall net xylem  $\text{Na}^+$  loading and accumulation in the shoot.

### **Material and Methods**

#### ***Plant material and growth conditions***

Wheat (*Triticum trugidum* L.) seeds (wild type – Tamaroi; lines conferring *Nax* loci – *nax1*, *nax2*, and *nax1:nax2* double line) were a kind gift of Dr Richard James (CSIRO Plant Industry, Canberra). Seeds were surface sterilised with 10% commercially available bleach (King White, Victoria, Australia) for ten minutes, rinsed thoroughly with deionised water, and then grown hydroponically for six days in the dark in aerated Basic Salt Medium (BSM) containing 0.1 mM CaCl<sub>2</sub> and 0.5 mM KCl (pH = 5.6; non-buffered).

#### ***Non-invasive ion flux measurements***

Net ion fluxes were measured using non-invasive ion-selective vibrating microelectrodes (the MIFE technique; University of Tasmania, Hobart, Australia). The principles of MIFE ion flux measurements are described in full elsewhere (Shabala *et al.* 1997), and all the details of microelectrodes fabrication and calibration are available in our previous publications (Shabala and Shabala 2002; Shabala *et al.* 2006). Liquid ionic exchangers used in this work were commercially available ionophore cocktails (60031 for K<sup>+</sup>; 71176 for Na<sup>+</sup>; 95297 for H<sup>+</sup>; all from Sigma).

#### ***Root K<sup>+</sup> and H<sup>+</sup> flux measurements***

One hour prior to measurement, six-day-old wheat seedlings were immobilised, with their roots placed horizontally in a 10 ml Perspex measuring chamber containing the bathing medium as described elsewhere (Bose *et al.* 2014; Chen *et al.* 2005). Two ion-selective microelectrodes, one for K<sup>+</sup>, another for H<sup>+</sup>, were used in the same experiment. Electrode tips were aligned and positioned 50 µm above the root surface. Once the steady-state fluxes were reached (40 to 60 min after the immobilisation), measurements have commenced. Net fluxes of ions were measured for 5 to 10 min from mature (~ 15-20 mm from the tip) root zone. Then salinity treatment (80 mM NaCl) was administered, and net K<sup>+</sup> and H<sup>+</sup> fluxes were measured for further 60 min.

### ***Root Na<sup>+</sup> flux measurements***

A so-called “recovery protocol” (Cuin *et al.* 2011) was used to quantify the activity of Na<sup>+</sup> efflux system in epidermal and stelar root tissues. Six-day-old wheat seedlings were treated with 150 mM NaCl for another 24 h in the darkness, leading to significant accumulation of Na<sup>+</sup> in root tissues. A seedling was then transferred to a 10 ml Perspex measuring chamber containing bathing medium with 150 mM NaCl. After 1 h adaption, the root was quickly but thoroughly rinsed with 10 mM CaCl<sub>2</sub> solution, to remove apoplastic NaCl. The root was then transferred into a clean chamber containing Na<sup>+</sup>-free BSM solution. Net ion fluxes were measured for approximate 60 min from either mature root epidermis (~ 15-20 mm from the tip), or root elongation zone. The first 20 min of recording was ignored during analyses, to eliminate the cofounding effect of the Donnan exchange in the cell wall (see Cuin *et al.* 2011 for justification and details).

For measurements from the xylem parenchyma, the root stele was mechanically isolated as described earlier (Shabala *et al.* 2010). An apical stelar segment was cut (the first 5-7 mm of the stele) and then immobilised in a Perspex chamber in BSM and left to recover for four to six hours in a presence of 50 mM NaCl. The recovery protocol (see above) was then applied to quantify activity of Na<sup>+</sup> efflux system at xylem parenchyma interface.

### ***RNA extraction and RT-qPCR experiments***

Total RNA was extracted from ~100 mg of roots from Tamaroi and *Nax* lines using the RNeasy Plant Mini Kit (Qiagen). First-strand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen), which includes the genomic DNA removal step. Relative transcript levels were assayed by real-time PCR analysis using Qiagen Rotor-gene real-time PCR system. The *TdSOS1* gene was amplified using two specific primers *TdSOS1*; 5'SOS (5'-ATTCCCTCAGGTGCTTCGTG-3') and 3'SOS (5'-TTTCCTCGAGCAACCCAGTC-3'). The wheat actin gene (Genebank Accession No. [AB181991.1](#)) was used as an internal control for gene expression. The actin primers were AF (5'-TACACGAAGCGACATACAA-3') and AR (5'-

AATAGAGCCACCGATCCA -3'). RT-qPCR conditions were as follows: 95 °C for 5 minutes, 94 °C for 30 s (50 cycles), 58 °C for 30 s and 72 °C for 1 min. Amplified products were detected using QuantiNova SYBR Green PCR Kit (Qiagen). Each data point was determined in triplicate in each sample and presented as mean  $\pm$  SE.

### *Statistical analysis*

Data were analysed using one-way of variance and treatment mean separations were performed using Duncan's multiple range tests at 5% level of significance in IBM Statistics 21.

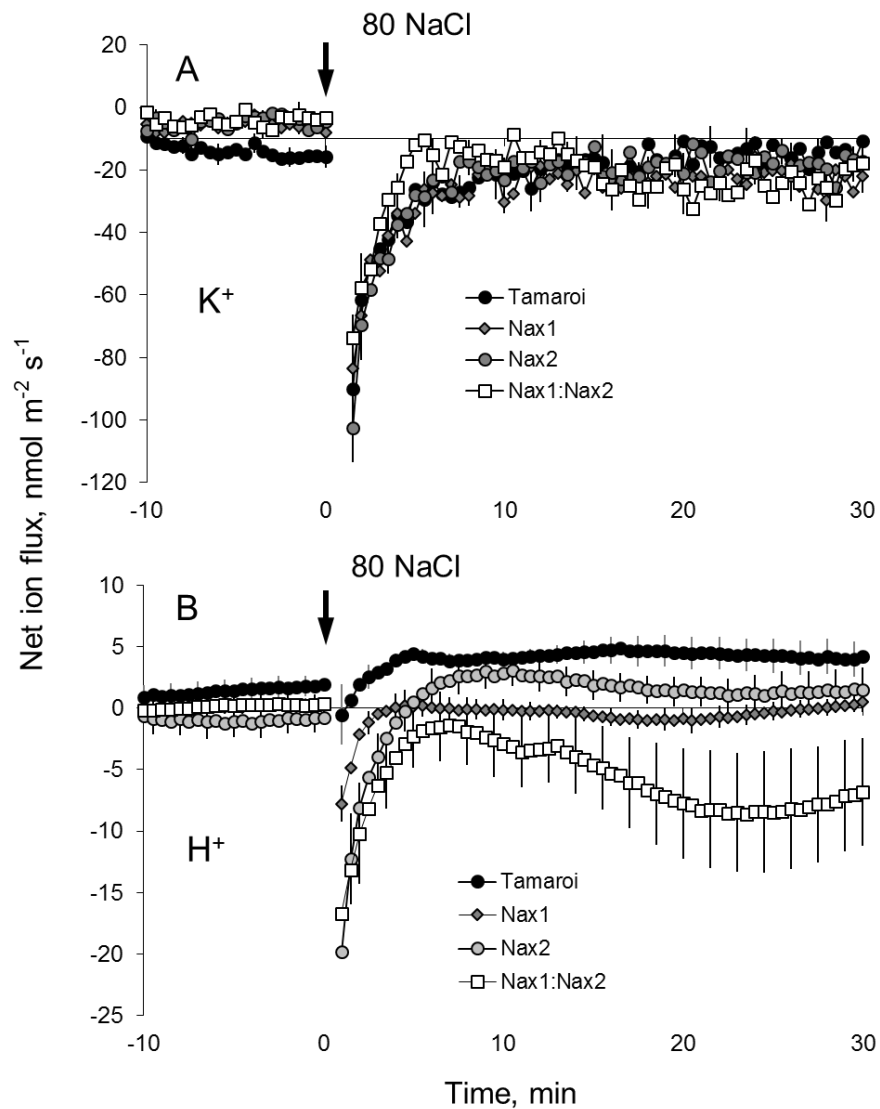
## **Results**

### *NaCl-induced ion flux response in root*

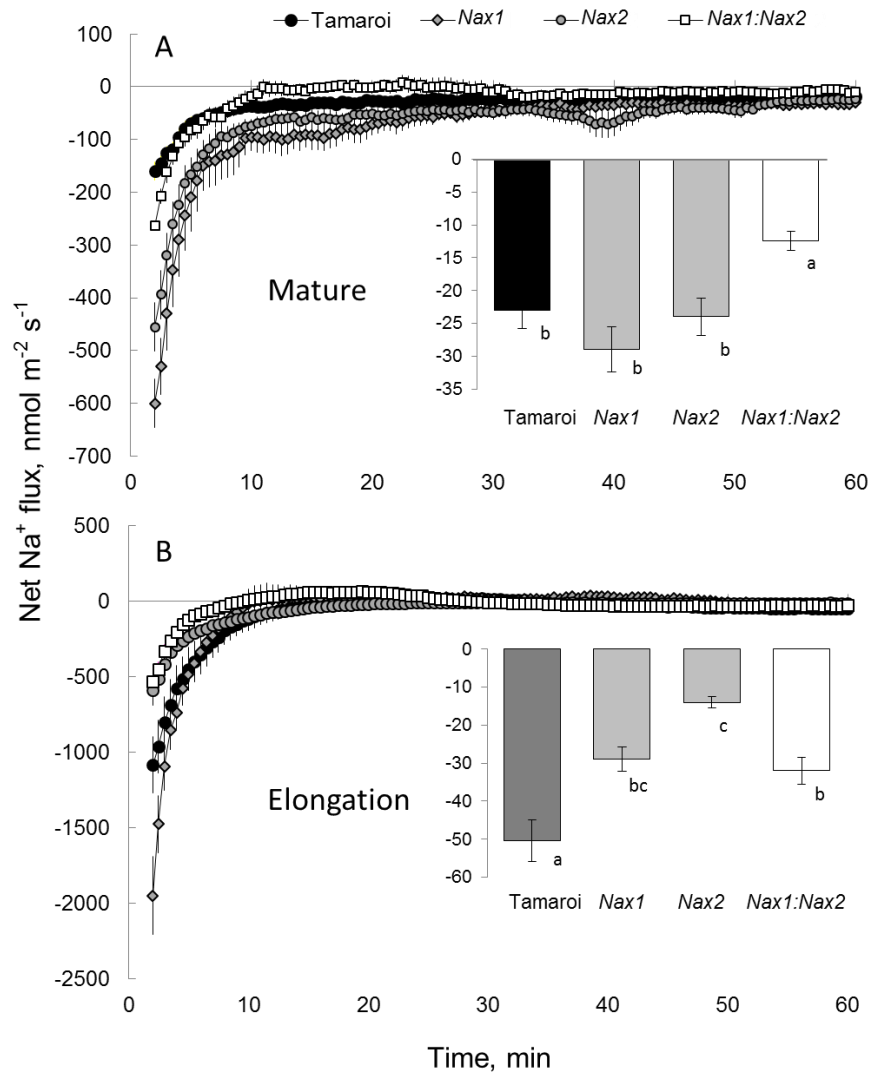
Addition of 80 mM NaCl caused significant changes in net ion ( $K^+$  and  $H^+$ ) fluxes from mature root zone of durum wheat (Tamaroi and *Nax* lines; Fig. 1). A peak  $K^+$  efflux was reached in several minutes after stress onset (Fig. 1A), followed by a gradual recovery of  $K^+$  flux (although it remained always negative, i.e. net efflux). No significant (at  $P < 0.05$ ) differences in NaCl-induced  $K^+$  efflux kinetics were found between Tamaroi and either of *Nax* lines. Salinity-induced  $H^+$  flux kinetics showed some genotypic specificity (Fig. 1B), with steady-state  $H^+$  influx/efflux values (measured 30 min after stress onset) being Tamaroi  $>$  *Nax1* = *Nax 2*  $>$  *Nax1:Nax2* lines (Fig. 1B). Interestingly, the  $H^+$  efflux was observed in *Nax1:Nax2* line, compared to the mild  $H^+$  influx found in Tamaroi and the other two *Nax* lines.

Transfer of salt-treated roots (150 mM NaCl for 24 h) into Na-free solution resulted in a significant  $Na^+$  efflux from root epidermis in both mature and elongation root zones (Fig. 2). Inserts in each panel denote mean total  $Na^+$  efflux over the last 30 min of measurements. No clear trends have emerged for mature zone (see insert in Fig. 2A); however,  $Na^+$  efflux in root elongation zone (where *SOS1* exchanges are predominantly expressed; Shi *et al.*, 2002) in all *Nax* lines

was significantly lower compared with parental Tamaroi line (significant at  $P < 0.05$ ).



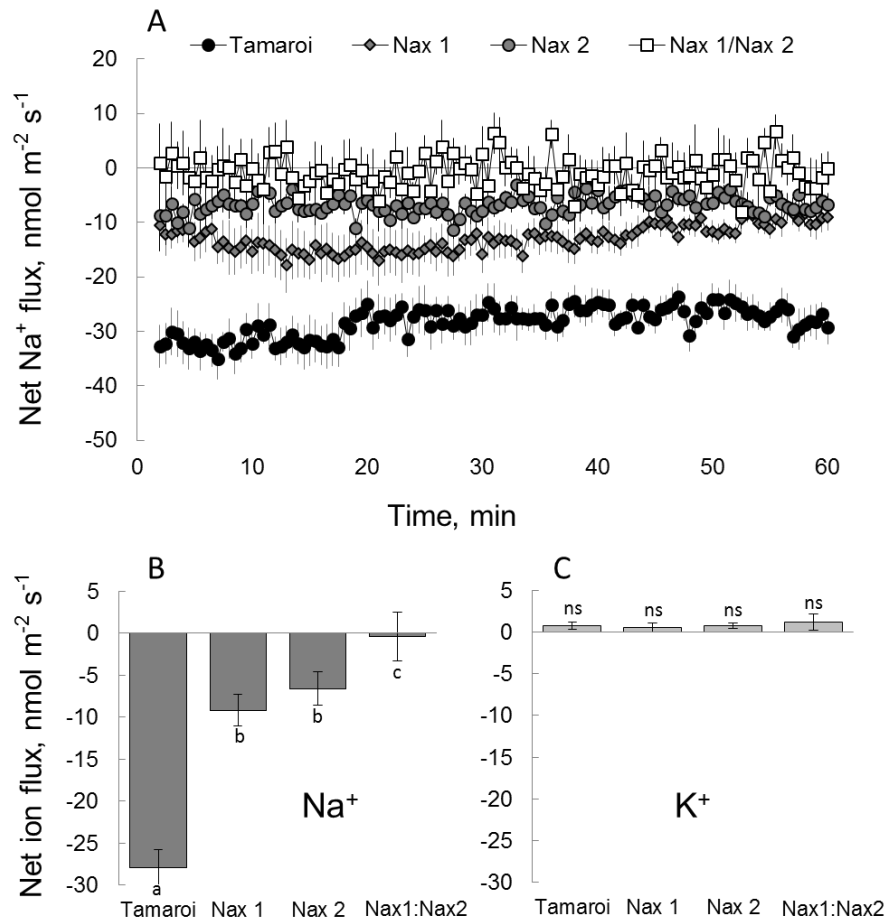
**Fig. 1.** Transient  $K^+$  (A) and  $H^+$  (B) flux responses measured from the mature zone of root epidermis from *Nax* lines and a parental line Tamaroi in response to acute 80 mM NaCl treatment. Mean  $\pm$  SE ( $n = 6$ ). For all MIFE measurements, the sign convention is “efflux negative”.



**Fig. 2.** Net Na<sup>+</sup> fluxes measured in “recover protocols” from mature (A) and (B) apical (elongation zone; ~ 2 mm from root tip) zones of epidermal root cells of *Nax* lines and a parental line Tamaroi after being transferred from 150 mM NaCl solution (24 h treatment) to sodium-free BSM . Mean  $\pm$  SE (n = 6). Inserts in each panel denote mean total Na<sup>+</sup> efflux over the first 60 min upon salt treatment.

To quantify the activity of Na<sup>+</sup> efflux system at the xylem parenchyma interface, root stele was mechanically isolated as described in the Materials and Methods, and net ion fluxes were measured after transferring stele from 50 mM NaCl (pre-treated for 6 hours) into a Na<sup>+</sup>-free BSM. Net Na<sup>+</sup> efflux in the parental line Tamaroi was significantly ( $P < 0.05$ ) higher compared with other

three genotypes, while *Nax1:Nax2* line has the lowest  $\text{Na}^+$  efflux. Net  $\text{K}^+$  fluxes were not significantly different from zero (Fig. 3C) indicating that the observed difference in measured  $\text{Na}^+$  flux was not an artefact originating from the possible poor discrimination of  $\text{Na}^+$  LIX between  $\text{Na}^+$  and  $\text{K}^+$  (see Chen *et al.*, 2005 for details).

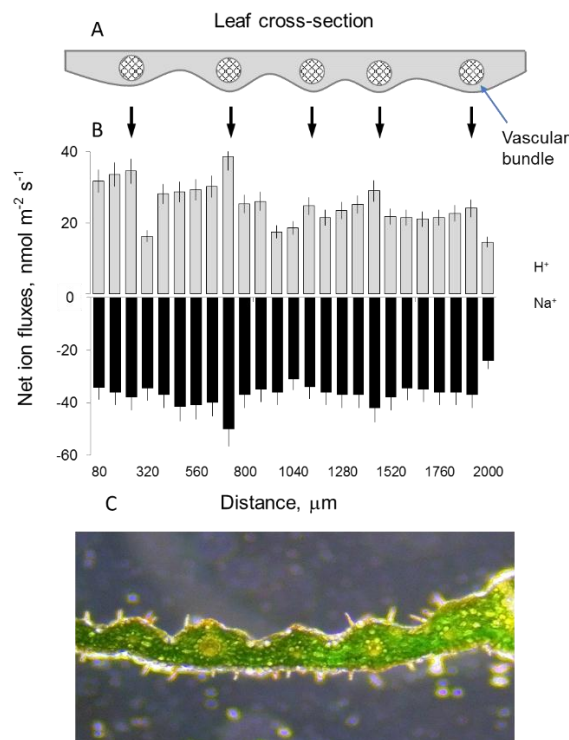


**Fig. 3.** A- Net  $\text{Na}^+$  fluxes measured in “recovery protocols” from the root stele after 6 h exposure to 50 mM NaCl. B, C – Mean  $\text{Na}^+$  (B) and  $\text{K}^+$  (C) values measured from stelar tissue over the 60 min interval after transferring stele to Na-free solution. Mean  $\pm$  SE (n = 6).

### *Ion flux profiles in leaf sheath and blade tissues*

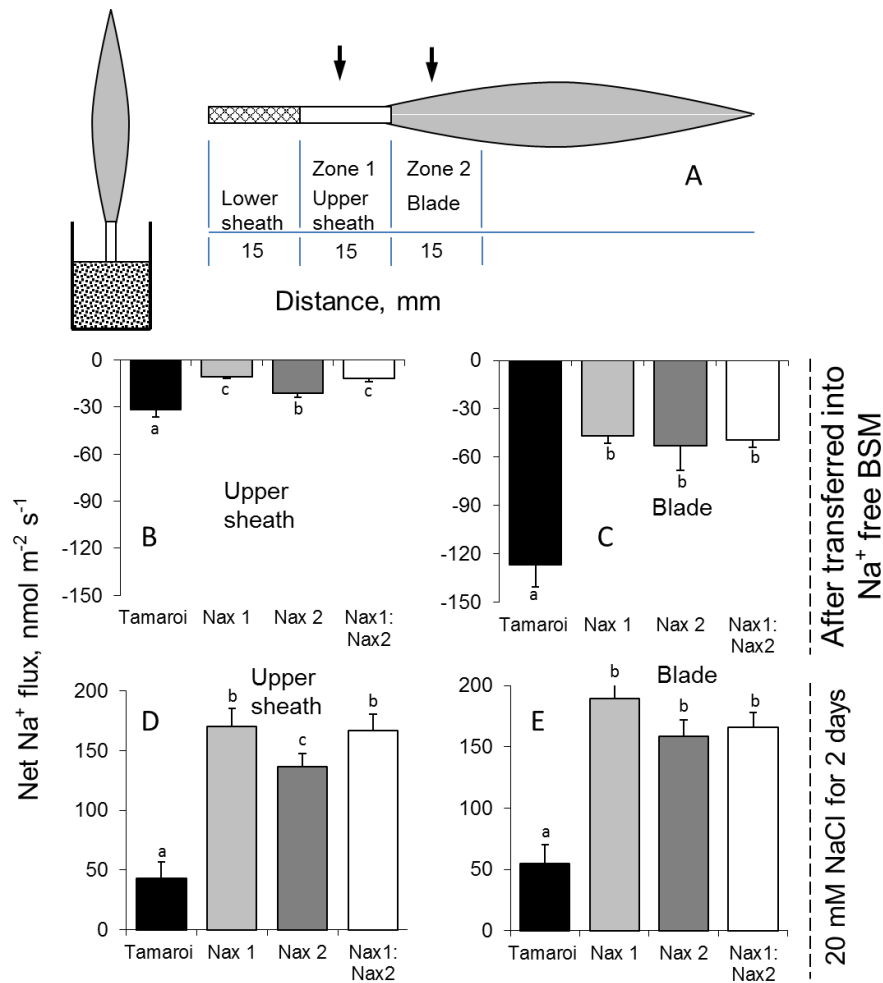
Past studies have pointed out at the leaf sheath as well as roots as a most likely location of *HKT1;4* gene expression conferred by *Nax1* loci (Huang *et al.*, 2006;

James et al. 2006). Accordingly, the difference in net ion fluxes between vascular bundles and leaf mesophyll in Tamaroi and *Nax* lines were compared. As *HKT* genes involved in  $\text{Na}^+$  retrieval from the xylem are believed to be expressed in tissues surrounding the vascular bundle (Horie *et al.*, 2009), it was very critical to ensure that electrodes are positioned exactly above this tissue. To ensure this, methodological experiments were conducted mapping cross-sectional ion flux profiles in wheat leaves (Fig. 4). Similar to our previous reports for mean mesophyll (Shabala *et al.*, 2002), ion flux profiles in wheat leaves showed strong correlation with leaf anatomy, with both the highest  $\text{H}^+$  influx and highest  $\text{Na}^+$  efflux occurring from vascular bundles (Fig. 4B). Thus, places with the highest  $\text{H}^+$  influx and  $\text{Na}^+$  efflux activity were then used to compare parental and *Nax* lines (depicted in Fig. 5).



**Fig. 4.** Ion flux profiles along the cross-section of wheat leaf. A – A schematic model depicting position of major veins in wheat leaf. B - Net  $\text{H}^+$  (grey bars) and  $\text{Na}^+$  (dark bars) fluxes measured at different parts of the leaf (please refer to panel A) from control plants in order to assess leaf profile. Mean  $\pm$  SE (n = 4 individual leaves). C – A microscopic image depicting a cross-section of wheat leaf.



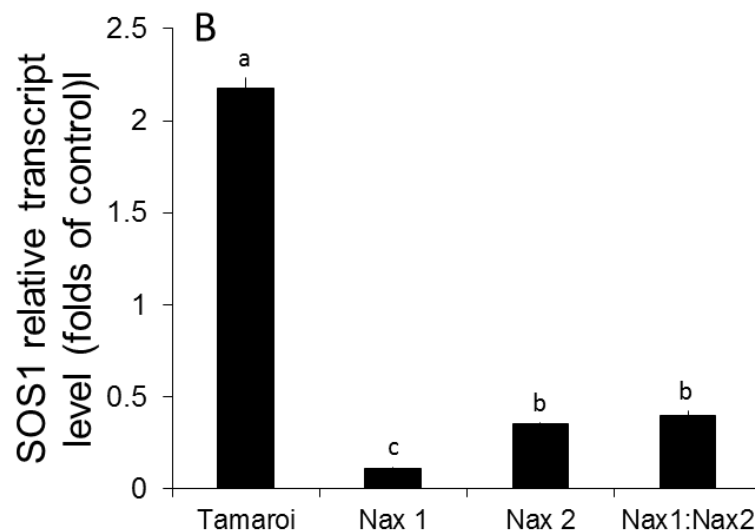


**Fig. 5.** Activity of Na<sup>+</sup> efflux systems in leaf blade and upper sheath tissues of wheat lines. A – A schematic diagram of the experimental protocol. Excised leaves were immersed in a beaker containing 20 mM NaCl solution with the sheath being immersed in the solution 15 mm deep and treated for 2 days. A special attention was paid to ensure that the depth of insertion and the total surface area of the leaf exposed to salinity was the same in all treatments. B, C – Net Na<sup>+</sup> fluxes measured from upper sheath (B, zone 1 in Panel A) and leaf blade tissue (C, zone 2 in Panel A) in the absence of salt treatment (with leaves being immersed in Na<sup>+</sup> free BSM solution). D, E – Net Na<sup>+</sup> fluxes measured from upper sheath (D) and leaf blade tissue (E) after leave being exposed to 20 mM NaCl for 2 days. Mean ± SE (n = 5)

When treated with 20 mM NaCl, Tamaroi leaves showed significantly lower net Na<sup>+</sup> uptake compared with all *Nax* lines (a 3-fold difference; Fig. 5D, E; significant at  $P < 0.05$ ) both in upper sheath and blade tissue. As the net flux measured is a sum of channel-mediated Na<sup>+</sup> uptake and transporter-mediated Na<sup>+</sup> efflux, leaf segments were then transferred into Na<sup>+</sup>-free media, to reveal the specific contribution of Na<sup>+</sup>-efflux systems. Consistent with the above data, net Na<sup>+</sup> efflux was much more pronounced in parental Tamaroi line compared with any of *Nax* lines in leaf blade tissue (Fig. 5B, C; significant at  $P < 0.05$ ). The same pattern was observed both in the upper sheath (Fig. 5D) and leaf blade (Fig. 5E) regions, although net Na<sup>+</sup> efflux was about 4-fold stronger from the leaf blade.

***Effect of Nax loci on SOS1 transcript level in roots***

Relative SOS1 transcript level was up-regulated (compared to control) in salt-treated (150 mM for 3 weeks) Tamaroi roots (2.18 folds of control; Fig. 6), while it was down-regulated in all *Nax* lines (0.11, 0.35, 0.39 fold of control for *Nax1*, *Nax2*, *Nnax1:Nax2* lines, respectively; all significant at  $P < 0.001$ ).



**Fig. 6.** The relative transcript level of TdSOS1 gene in root in Tamaroi and *Nax* lines (6-day old seedlings exposed to 150 mM NaCl for 24 h). Each data point was determined in six replicates in each sample and presented as mean  $\pm$  SE.

### **Discussion**

Until now, *Nax* loci were associated with hosting candidate genes responsible for retrieval  $\text{Na}^+$  from the xylem and restricting its transport from root to leaves (James *et al.* 2006; Byrt *et al.* 2014). Here we show that *Nax* loci also affect SOS1-like  $\text{Na}^+/\text{H}^+$  exchanger expression and activity in wheat.

### ***Activity of SOS1-like $\text{Na}^+/\text{H}^+$ exchanger in root epidermis is suppressed in *Nax* lines***

As shown in Fig. 1B and Fig. 2B, net  $\text{H}^+$  influx and  $\text{Na}^+$  efflux in root epidermal cells in *Nax* lines were much lower than those in Tamaroi. These observations are fully consistent with the notion that *Nax* loci also affect SOS1-like  $\text{Na}^+/\text{H}^+$  exchanger activity in root epidermis. First, the observed patterns were found only in the root apex (where SOS1 transporters are expressed; Shi *et al.* 2002) but not in mature root zone (Fig. 2). Second, measured  $\text{Na}^+$  flux was sensitive to amiloride, a known inhibitor of  $\text{Na}^+/\text{H}^+$  exchanger (data not shown; but see Cuin *et al.* 2011 for supporting evidence). Contrary to animals, higher plants lack ATP-driven  $\text{Na}^+$ -pumps, and rely on  $\text{Na}^+/\text{H}^+$  exchanger to exclude cytotoxic  $\text{Na}^+$  back to apoplast. It is estimated that a typical glycophyte plant such as barley exclude between 95 and 98% of total  $\text{Na}^+$  that entered the cell (Munns 1985); this could be partly achieved by operation of SOS1 plasma membrane  $\text{Na}^+/\text{H}^+$  exchanger, which has been addressed in Arabidopsis (Shi *et al.* 2002; Shi *et al.* 2003). Overexpression of *AtSOS1* has been shown to improve salt tolerance in transgenic Arabidopsis (Shi *et al.* 2003) and *OsSOS1* has been characterized in rice and demonstrated a capacity for  $\text{Na}^+/\text{H}^+$  exchanger in plasma membrane vesicles of yeast (*Saccharomyces cerevisiae*) cells and reduced their net cellular  $\text{Na}^+$  content (Martinez-Atienza *et al.* 2007). Once the activity of SOS1 exchanger is suppressed under saline conditions, the  $\text{Na}^+$  exclusion and  $\text{H}^+$  uptake in root epidermis will be disturbed. This is what is observed here for all *Nax* lines.

### ***SOS1-like exchanger plays a substantial role in xylem Na<sup>+</sup> loading in wheat and its activity is reduced in stelar tissues of Nax lines***

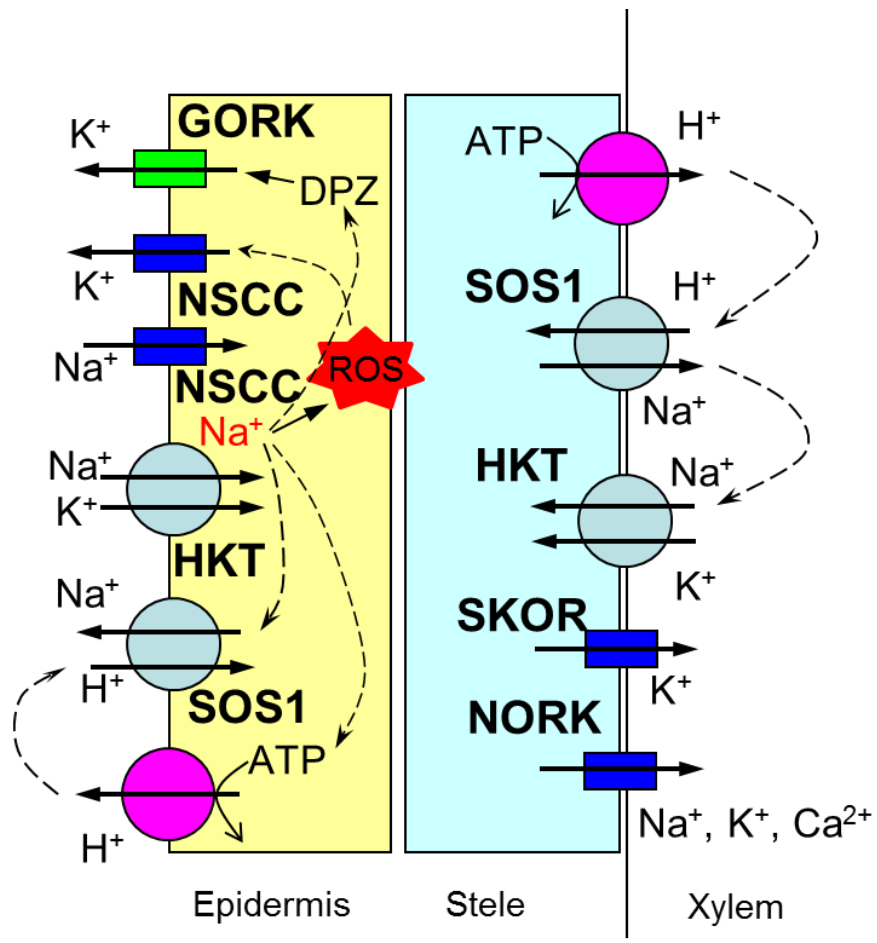
In Arabidopsis, SOS1 genes were found to be preferentially expressed in stelar root tissues (Shi *et al.* 2002) and proposed to operate in xylem Na<sup>+</sup> loading (Shi *et al.* 2002). Our findings of net Na<sup>+</sup> efflux being significantly higher in Tamaroi compared with any *Nax* lines (Fig. 3) are consistent with these suggestions and also provide a strong supportive evidence for SOS1-like exchanger being inhibited by *Nax* loci. Qualitatively similar patterns were observed in both upper sheath (Fig. 5B) and blade in leaf (Fig. 5C). Inevitably, a certain amount of Na<sup>+</sup> will overcome “the first defend line” (Na<sup>+</sup> exclusion from root epidermis) and enter the xylem. Here, it will be re-allocated by two pathways: 1) retrieved back into the stele by HKT transporters; or 2) transported to the shoot to be compartmentalized by leaf vacuoles for the purpose of osmotic adjustment. Tamaroi was observed to have more Na<sup>+</sup> loading into xylem due to the normal function of SOS1 while *Nax* lines had significantly less Na<sup>+</sup> loading in xylem. Thus, it appears that *Nax* loci confer two highly complementary mechanisms, both contributing to the same aim, namely reducing xylem Na<sup>+</sup> content. One of them is enhanced retrieval of Na<sup>+</sup> back into the root stele (as reported elsewhere; Blumwald *et al.* 2000; Shi *et al.* 2002), and another one – reduced rate of Na<sup>+</sup> loading into the xylem in the first instance (reported here). It may be speculated that such duality may play important adaptive role and provide more flexibility to plants. Indeed, as shown in this work *Nax* loci may suppress activity of SOS1-like Na<sup>+</sup>/H<sup>+</sup> exchanger in both epidermal (Fig. 2) and stelar (Fig. 3) tissue. So, on one hand this suppression reduces plant ability to exclude Na<sup>+</sup> from uptake. Yet, at the same time the rate of Na<sup>+</sup> entering the xylem is also reduced. This should result in more Na<sup>+</sup> staying in roots, either for osmotic adjustment (Shabala and Lew 2002), or salt stress-signalling (Wu *et al.* 2015) purposes. Also, from general point of view, it may always be beneficial for plants to have another “backup” mechanism when challenged by salinity stress, in case one of the system fails to operate.

The reduced SOS1-like activity in *Nax* lines could be explained (at least partially) by the reduced level of SOS1-transcript as revealed by RT-qPCR

experiments (Fig. 6). The downregulation of *TdSOS1* in *Nax* line root under saline condition could explain why the function of SOS1 exchanger was altered both in root epidermis and stele, compared with the parental line Tamaroi. It may be speculated that *Nax* loci might confer some “regulating genes” which have negative feedback on the *TdSOS1* expression at transcription level. This issue has to be addressed in a separate study.

### ***Root ion homeostasis under saline conditions: an improved model***

Based on our reported results, the following model can be suggested (Fig. 7).  $\text{Na}^+$  will enter the cell via either non-selective cation channels (NSCC, Demidchik and Maathuis 2007) or HKT transporters (Horie *et al.* 2001; Laurie *et al.* 2002; Garciadeblas *et al.* 2003; Haro *et al.* 2005; Horie *et al.* 2007), depolarizing plasma membrane and resulting in substantial  $\text{K}^+$  leak from root epidermis (Fig. 1) mediated by GORK channels (Anschutz *et al.* 2014; Pottosin and Shabala 2014). Increased cytosolic  $\text{Na}^+$  may lead to accumulation of ROS (Vass *et al.* 1992; Allakhverdiev *et al.* 2002), further exacerbating  $\text{K}^+$  efflux from cytosol via ROS-activated  $\text{K}^+$  permeable NSCC (Pottosin and Shabala 2014). No difference in any of the above mechanisms exists between Tamaroi and *Nax* lines. The major bulk of  $\text{Na}^+$  is excluded via plasma-membrane based SOS1  $\text{Na}^+/\text{H}^+$  exchanger fuelled by  $\text{H}^+$ -ATPase. *Nax* loci suppresses (directly or indirectly) the transcript level of SOS1 gene (Fig. 6) and its activity (Fig. 2). Part of  $\text{Na}^+$  accumulated in root cortex is then loaded into xylem. This loading is mediated by both passive (at early stages of salt stress; (Shabala *et al.* 2013) and active (SOS1-mediated) transport systems. In *Nax* lines the rate of  $\text{Na}^+$  loading is suppressed, either at transcriptional or a functional level (or both). Some of the loaded  $\text{Na}^+$  is taken back by HKT transporters located at xylem parenchyma interface (Munns and Tester 2008; Horie *et al.* 2009); this ability is much more pronounced in *Nax* lines. As a result, xylem  $\text{Na}^+$  loading is controlled by two highly complementary uptake and release systems providing plant with a greater versatility to respond to changing environment and control  $\text{Na}^+$  delivery to the shoot.



**Fig. 7.** A model depicting mechanisms contributing to root ion homeostasis under saline conditions. See text for details.

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## **Chapter 6: General discussion and future research**

### **General discussion**

Plant salinity tolerance is a polygenic trait, involving multiple mechanisms, with the contribution of genetic, development, and physiological interactions within the plant, in addition to interaction between the plant and its environment. The complex nature of plant salinity tolerance, the shortage of reliable and comprehensive screening methods, and the lack of a comprehensive understanding of the underlying molecular mechanisms of salinity tolerance hinder a further improvement in selecting and breeding for salt-tolerant crop species. This study goes towards elucidating the mechanism of salt tolerance in cereal crops. Research into whole-plant and molecular response to salinity has shed some light on several aspects of salt tolerance in barley and wheat.

There is sufficient variability within the existing barley (*Hordeum vulgare* and *Hordeum spontaneum*) and wheat (*Triticum aestivum* and *Triticum turgidum*) genotypes to potentially achieve the improvement of salinity tolerance using selection with a number of physiological parameters. We also showed that traits that are best for mild stress may be different from that for severe salinity stress. Hence, stomatal characteristics (stomatal conductance and stomatal density) and tissue ionic relations following the 5-week treatment with 200 mM NaCl can be used as reliable screening indicators for the breeding of mild-salinity tolerant barley genotypes; however, this reliability has to be validated in the real field. After all, the controlled condition (glasshouse) has various issues and limitations and could not mimic the practical conditions in the field. Only combined with field evaluations, these techniques can work better. These results also indicate the importance of increasing stomata density as an adaptive tool to optimise efficiency of CO<sub>2</sub> assimilation under moderate saline conditions, as well as benefits of the predominant use of inorganic osmolytes for osmotic adjustment, in barley. Another interesting observation was that wild barleys showed rather different strategies when dealing with salinity, as compared with cultivated

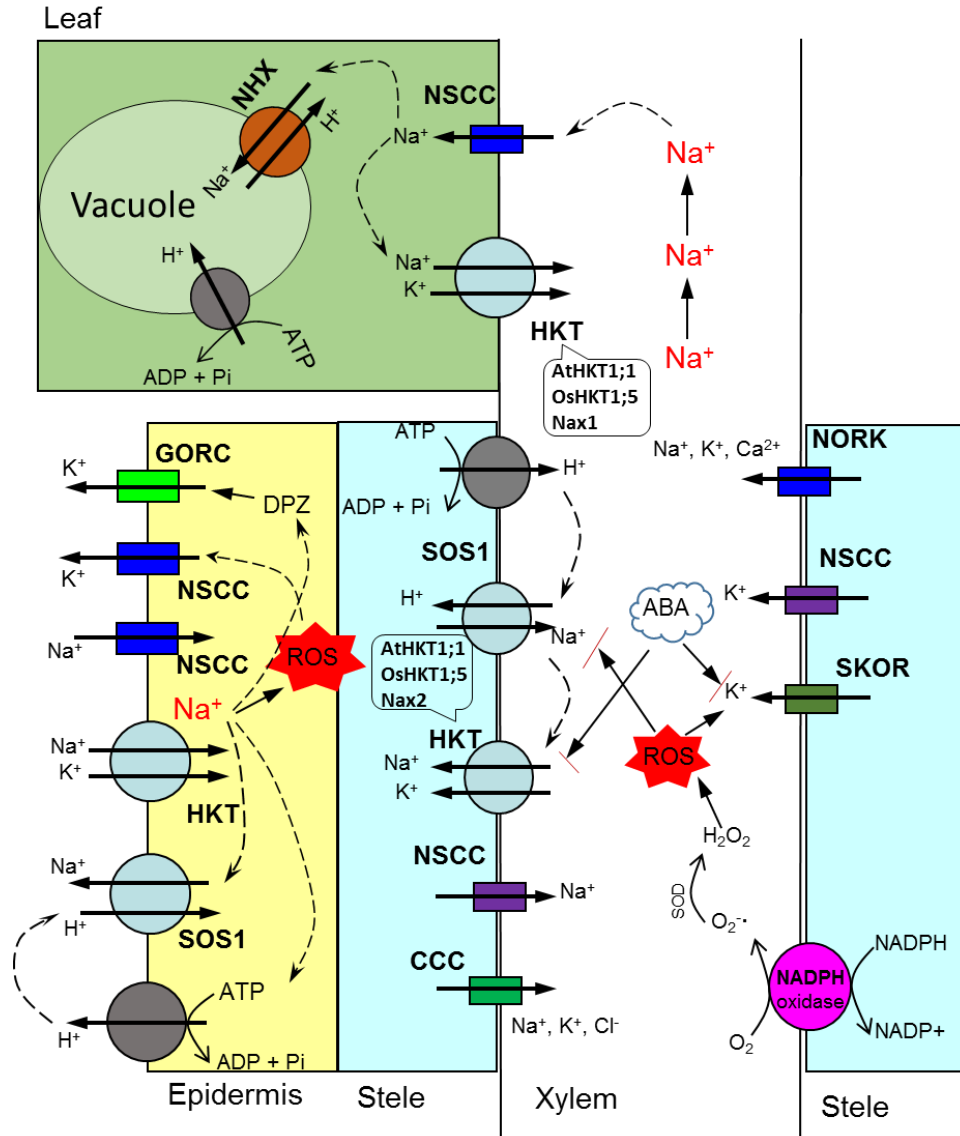
varieties. Given the superior salinity stress tolerance in wild barley, this warrants a need for more active research in this direction.

Among wheat species, durum wheat is generally less tolerant to salt stress than bread wheat (Munns *et al.* 2006; Munns and Tester 2008), mainly because of high rates of  $\text{Na}^+$  accumulation and poor  $\text{K}^+/\text{Na}^+$  discrimination (Asch *et al.* 2000; Chen *et al.* 2007a; Genc *et al.* 2007). In addition to  $\text{Na}^+$  exclusion, a large number of other mechanisms contribute to salt tolerance, such as stomatal characteristics and the accumulation of compatible solutes in leaf (Brugnoli and Lauteri 1991; Rivelli *et al.* 2002; Chen *et al.* 2007b). In this project, a large number of wheat (*Triticum aestivum* and *Triticum turgidum*) cultivars were screened by using a broad range of physiological indices and the aforementioned difference between bread and durum wheat in response to salinity stress was affirmed. In general, leaf  $\text{K}^+/\text{Na}^+$  ratio and  $\text{K}^+$  in leaf and xylem were the major factors determining salinity tolerance in wheat. Most of the bread wheats showed better  $\text{Na}^+$  exclusion that was associated with higher relative yield. In durum wheat, maintaining high leaf and xylem  $\text{K}^+$  content made the strongest contribution to salt tolerance. It appears that  $\text{Na}^+$  sequestration in durum wheat and  $\text{Na}^+$  exclusion in bread wheat following the 5-week treatment with 150 mM NaCl can be used as reliable screening indicators for salt tolerance. We also concluded that plant survival scores under high salt stress can be used in bread wheat as a preliminary selection for  $\text{Na}^+$  exclusion gene(s).

This work has also advanced our understanding of the molecular and physiological mechanisms mediating  $\text{Na}^+$  uptake and its delivery to the shoot. Based on our reported results, an improved model was suggested (Fig. 1). When plants subject to salinity stress, massive  $\text{Na}^+$  will be transported into cells through NSCC (Demidchik and Maathuis, 2007) or HKT (Horie *et al.*, 2001; Laurie *et al.*, 2002; Garciadeblas *et al.*, 2003; Haro *et al.*, 2005; Horie *et al.*, 2007). This will cause the depolarization of plasma membrane, and then a substantial leak  $\text{K}^+$  from root epidermal cells, which is mediated by GORK channels (Anschutz *et al.*, 2014; Pottosin and Shabala, 2014).

The Na<sup>+</sup>-induced-accumulation of ROS in the cytosol (Allakhverdiev *et al.*, 2002; Vass *et al.*, 1992) further exacerbates K<sup>+</sup> efflux from the cytosol via ROS-activated K<sup>+</sup> permeable NSCC (Pottosin and Shabala, 2014). SOS1 Na<sup>+</sup>/H<sup>+</sup> exchanger localised at the plasma membrane is responsible for most of Na<sup>+</sup> exclusion. Reduction of Na<sup>+</sup> loading into the xylem via SOS1 has been affirmed to be the complementary mechanism of reducing xylem Na<sup>+</sup> content (the other pathway is enhanced retrieval of Na<sup>+</sup> back into the root stele via HKT; conclusions from Chapter 5). Eventually, part of Na<sup>+</sup> accumulated in the root cortex is then loaded into xylem, which is mediated by both passive (at early stages of salt stress; Shabala *et al.*, 2013) and active (SOS1-mediated) transport systems. Some of loaded Na<sup>+</sup> is taken back by HKT transporters located at the xylem parenchyma interface (Munns and Tester, 2008; Horie *et al.*, 2009). Consequently, there two highly complementary uptake and release systems controlling xylem Na<sup>+</sup> loading, providing plant with a greater versatility to respond to changing environment and control Na<sup>+</sup> delivery to the shoot.





**Fig. 1.** The model depicting molecular mechanisms underlying regulation of Na<sup>+</sup> transport from root to shoot.

The inevitable accumulation of Na<sup>+</sup> in the xylem under salt stress will cause the generation of ROS, which could stimulate NADPH-oxidase to produce more H<sub>2</sub>O<sub>2</sub>. Further supporting evidence showed that H<sub>2</sub>O<sub>2</sub> accumulation induced by activation of NADPH-oxidase can facilitate xylem K<sup>+</sup> loading. This process could be mediated by SKOR, a stellar K<sup>+</sup> outward rectifier (V éry *et al.* 2014) which is known to be expressed at the xylem parenchyma interface (Wegner and De Boer, 1997) and mediate potassium delivery to the shoot. At the same time, NADPH oxidase-mediated apoplastic H<sub>2</sub>O<sub>2</sub> production acts upstream of the xylem Na<sup>+</sup> loading and

could prevent  $\text{Na}^+$  loading into xylem. Previous studies (Demidchik and Maathuis 2007) have implicated NADPH oxidase as a major source of apoplastic ROS production and the upstream component of net  $\text{Ca}^{2+}$  uptake in root epidermis. In Chapter 4, we showed that inhibition of NADPH oxidase by DPI has significantly reduced the magnitude of  $\text{H}_2\text{O}_2$ -induced  $\text{Ca}^{2+}$  ion fluxes measured from root stele (Fig. 8 & 9; Chapter 5). This is fully consistent with the idea of NADPH oxidase acting upstream of  $\text{Ca}^{2+}$  signalling in stelar tissues in both control (Fig. 8A; Chapter 5) and salt-treated plants (Fig. 9A; Chapter 5). NADPH oxidase is known to be activated by cytosolic  $\text{Ca}^{2+}$  (Lecourieux *et al.* 2006), forming a positive feedback loop. The inhibition of channel-mediated efflux of  $\text{K}^+$  and  $\text{Cl}^-$  into the xylem by ABA has been well documented (Cram and Pitman, 1972; Roberts, 1998; Gilliham, 2002). Besides, it was observed in this study that ABA could attenuate the activity of retrieval of  $\text{Na}^+$  from xylem. The possible reason for that may be the fact that addition of ABA stimulated  $\text{H}^+$  extrusion into xylem (Clarkson and Hanson, 1986), affecting activity of SOS1  $\text{Na}^+/\text{H}^+$  antiporter at the plasma membrane of xylem parenchyma cells. Thus, it appears that control of xylem ion loading and the maintenance of the optimal K/Na ratio in the xylem sap is achieved by the orchestrated action of ROS, ABA, and control of membrane voltage by  $\text{H}^+$ -ATPase. More studies are needed to reveal the mechanisms of xylem ion loading and kinetics of this regulation. With the related characterized genes, the beneficial trait could be utilized by breeders to generate the salt-tolerant plant. For instance, overexpression of the specific ion transporter gene expressing only in the early stage of salinity and responsible for loading  $\text{Na}^+$  into xylem will be valuable for osmotic adjustment, while silencing those genes which only appear in the later stage of salinity stress and also could regulate xylem  $\text{Na}^+$  loading will be beneficial to avoidance of  $\text{Na}^+$  over-accumulation in shoot.

Once  $\text{Na}^+$  enters into cytosol through NSCC (Demidchik and Tester 2002; Shi *et al.* 2002; Demidchik and Maathuis 2007), part of them is sequestered by NHX (Blumwald *et al.* 2000) while a certain proportion is retained in leaf ligule (Byrt *et al.*, 2014). Both of these processes can promise the normal physiological metabolism. However, in barley, there is a need to match xylem  $\text{Na}^+$  loading capacity with the ability of mesophyll cell to sequester  $\text{Na}^+$  in vacuole. As

aforementioned in Chapter 4, the strategy for tolerant barley genotypes to cope with excessive  $\text{Na}^+$  in the beginning of salinity stress is to transport  $\text{Na}^+$  to shoot and sequester into vacuole for osmotic adjustment. This requires coordination with ‘transpiration pull’ and stomatal control. The findings that stomata density correlated positively with salinity tolerance (Fig. 10A; Chapter 2) and  $\text{Na}^+$  delivery to the shoot has a positive correlation with the ‘transpiration pull’ ( $g_s$  values; insert in Fig. 9C; Chapter 4), reflect an intrinsic cascade of  $\text{Na}^+$  transport from root to shoot in the early stage of salinity stress.

### **Future research**

This project was an integral part of the GRDC-funded collaboration between Australia and China on barley and wheat genetic resources. Among studied barley genotypes, the cultivar SYR01 was found to be the most salinity tolerant, with higher leaf tissue tolerance, better  $\text{K}^+$  retention and lower xylem  $\text{Na}^+$  loading. Naso Nijo and Unicorn were found to be most sensitive to salinity stress. These genotypes could be used as the parent lines for DH population/Near Isogenic Lines to find out the QTLs/genes conferring salinity tolerance mechanisms. Among bread wheat, Westonia and Persia 6 were respectively regarded as the most tolerant and sensitive to salt stress; while Tehuacan 60 and Odin were the most tolerant and sensitive to salinity stress in durum wheat. It is recommended that these contrasting genotypes are used in further physiological (e.g. mechanisms) and genetic (QTL mapping) studies to improve salinity stress tolerance in wheat.

Significant breakthroughs have been made on the mechanisms of salinity tolerance. Nevertheless, several questions still remain unknown in our knowledge:

1. Given the generally negative perception of GM crops by public, it will be important to reveal the QTLs for the major functional traits contributing to salinity tolerance and, specifically, for those mechanisms involved in the osmotic adjustment. It will be also essential to reveal the nature of possible

interactions between different mechanisms and reveal the molecular identity of signals underlying these interactions.

2. In this work, a part of signalling pathways involved in xylem ion loading have been revealed. However, it will be rather naïve to assume that regulation of xylem ion loading is limited to ABA and H<sub>2</sub>O<sub>2</sub>. So, what are other signal molecules controlling this process?
3. While signalling pathways responding to salinity stress at the intracellular level have been well described, more attention has to be paid to the long-distance signalling. How do leaves know that roots are in the saline soil, when salt is delivered in the xylem to leaves?
4. Finally, once the key transport mechanisms (and related genes) are identified, breeders will need to face a challenge of combining them together in one ideotype to produce a real salt-tolerant crop.

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